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DIFFUSION PROPERTIES OF DENTAL PLAQUE

IN RELATION TO DENTAL CARIES

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PREFACE

This research was conducted in the Department of Oral Medicine and Pathology, Glasgow Dental Hospital and School, during the period January 1976 to January 1980 while the author was employed as a Basic Grade Physicist in the Department of Clinical Physics and Bio-Engineering, West of Scotland Health Boards, 11 West Graham Street, Glasgow G4 9LF.

This thesis contains original research work conducted by the author personally, with some assistance from technical staff (as detailed in the Acknowledgements) under the direct supervision of the author. Dr. D. A. M. Geddes collected the plaque samples for the "Stephan curve" experiments described in Chapter 9 and other clinical colleagues collected the plaque samples used in other experiments.

Parts of the work reported in this thesis have been published, or submitted for publication, in scientific journals or have been presented at scientific conferences, as follows:-

.....
A. Publications in journals

Measurement of the rate of diffusion of radioactive xenon in human dental plaque in vitro. S. G. McNee, D. A. M. Geddes, C. Main and F. C. Gillespie. Archives of Oral Biology, 1979, 24: 359-362.

Measurements of the diffusion coefficient of NaF in human dental plaque in vitro. S. G. McNee, D. A. M. Geddes, C. Main and F. C. Gillespie. Archives of Oral Biology 1980, 25: 819-823.

Diffusion of sugars and acids in human dental plaque in vitro. S. G. McNee, D. A. M. Geddes and D. A. Weetman. Archives of Oral Biology. Submitted, March, 1981.

Effect of extracellular polysaccharides on diffusion of NaF and ¹⁴C-sucrose in dental plaque and in sediments of Streptococcus sanguis 804 (NCTC 10904). S. G. McNee, D. A. M. Geddes, D. A. Weetman, D. Sweeney and J. A. Beeley. Archives of Oral Biology. Submitted, March, 1981.

B. Presentations at conferences

Diffusion of radioactive xenon in plaque in vitro. S. G. McNee, C. Main, D. A. M. Geddes and F. C. Gillespie. 26th Meeting, British Division I.A.D.R., London, 1978.

Diffusion of NaF in dental plaque in vitro. S. G. McNee, D. A. M. Geddes, C. Main and F. C. Gillespie. 25th ORCA Congress, Turku, Finland, 1978.

The effect of 0.2% NaF rinses daily on human plaque acidogenicity in situ. D. A. M. Geddes and S. G. McNee. 25th ORCA Congress, Turku, Finland, 1978.

Measurement of the diffusion coefficient of Na^+ and F^- in dental plaque over the pH range 4 to 8. S. G. McNee, D. A. M. Geddes, C. Main and F. C. Gillespie. 27th Meeting, British Division I.A.D.R., Sheffield, 1979.

Diffusion of sucrose in dental plaque and the influence of glucan on diffusion of NaF. S. G. McNee, D. A. M. Geddes, D. Sweeney, D. A. Weetman and J.A. Beeley. 26th ORCA Congress, Stirling, Scotland, 1979.

Assessment of the diffusion characteristics of dental plaque. S. G. McNee, D. A. M. Geddes, D. Sweeney, D. A. Weetman and J. A. Beeley. 28th Meeting, British Division I.A.D.R., Leeds, 1980.

The permeability of dental plaque. S. G. McNee, D. A. M. Geddes, D. Sweeney, D. A. Weetman and J. A. Beeley. 27th ORCA Congress, Marburg, West Germany, 1980.

SUMMARY

Diffusion in dental plaque is recognised to be important to caries formation. It has been postulated that plaque acts as a barrier to diffusion, thereby trapping harmful acid at the tooth surface. Extracellular polysaccharides, such as glucan, in the plaque matrix were thought to be the diffusion controlling component of plaque. However, there were no quantitative measurements to support these hypotheses.

The aim of this study was to quantify rates of diffusion in plaque from individual subjects, using experimental techniques which would circumvent the effects of chemical and metabolic reactions. Initial measurements were made using the radioisotope ^{133}Xe of the inert gas xenon. Plaque was sampled after 24 h accumulation and layered into a brass dish to give a disc of plaque of known, uniform thickness which was then equilibrated in a gas-tight chamber containing ^{133}Xe . Clearance of ^{133}Xe from the plaque into a ^{133}Xe -free environment was then monitored and found to be single-exponential. The diffusion coefficient, D (cm^2s^{-1}), was calculated using the equation $D = 0.2809 L^2/T_{\frac{1}{2}}$, where L is the thickness of the sample (cm) and $T_{\frac{1}{2}}$ (s) is the clearance halving-time i.e. the time for the average concentration within the plaque to decrease two-fold.

The mean value of D , \pm S.D., for xenon in plaque at 37°C was $(7.2 \pm 0.6) \times 10^{-6} \text{ cm}^2\text{s}^{-1}$, which is only two-fold lower than D for xenon in water at 37°C . Values of D in plaque were calculated for O_2 and CO_2 using Graham's law of diffusion. Values of $T_{\frac{1}{2}}$ for a $100 \mu\text{m}$ thick layer of plaque would be 3.9, 1.9 and 2.2 s for Xe,

O_2 and CO_2 , respectively. It was concluded that plaque does not form a significant barrier to the diffusion of these small, uncharged species.

The technique was adapted to measure D for NaF in plaque samples held in a brass dish with a Millipore filter. After equilibration of the sample in buffer containing NaF, the clearance of Na and F from the sample into NaF-free buffer was monitored. The mean D for NaF in plaque at $37^\circ C$, \pm S.D., was $(4.2 \pm 0.5) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, which is about one quarter of D for NaF in water at $37^\circ C$. Na and F diffused at the same rate and D for NaF did not vary over the physiological pH range of plaque. The $T_{\frac{1}{2}}$ for NaF in a $100 \mu\text{m}$ thick layer of plaque would be 6.7 s, showing that diffusion of NaF in thin layers of plaque is rapid. Thus, a thorough cleaning and polishing of the teeth may not be essential before topical application of fluoride.

Diffusion coefficients were measured for sugars and acids in plaque samples in which metabolic activity was prevented by fixation with glutaraldehyde. It was shown that rates of diffusion of NaF and xylitol (which is not rapidly metabolised in plaque) were not significantly affected by fixation of the plaque. Mean values of D , \pm S.D., for ^{14}C -sucrose, sodium acetate and sodium lactate in glutaraldehyde-fixed plaque at $37^\circ C$ were $(3.0 \pm 0.3) \times 10^{-6}$, $(5.0 \pm 0.7) \times 10^{-6}$ and $(4.8 \pm 0.3) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, respectively. Acetate and lactate diffused three-fold, and sucrose about two-fold more slowly in plaque than in water. Values of $T_{\frac{1}{2}}$ for a $100 \mu\text{m}$ layer of plaque would be 9.4, 5.6 and 5.9 s for the sucrose, acetate and lactate, respectively. It was concluded that acids and

sugars diffuse rapidly in plaque, with acetic and lactic acids diffusing faster than sucrose. Thus, the prolonged period of low plaque pH following exposure to sucrose (Stephan curve) is probably due to continued acid production in the plaque.

The effect of glucan on diffusion was assessed using sediments of Streptococcus sanguis 804 (NCTC 10904) incubated initially with glucose, to produce glucan-free cultures, and then with sucrose, to produce glucan-containing cultures. D for NaF was positively correlated with total carbohydrate concentration in the sediment, with diffusion in cell-free glucan two-fold faster than in glucan-free sediments. For sediments with total carbohydrate concentrations similar to those reported for plaque, there was only a small (25-35 per cent) increase in diffusion rates for NaF and for ¹⁴C-sucrose with increasing carbohydrate concentration. In individual plaque samples from 15 subjects, D for NaF was also found to be positively correlated with total carbohydrate concentration although the coefficient of variation for D was small (12 per cent). Incubation of three plaque samples in sucrose-containing broth led to a three- to five-fold increase in total carbohydrate concentration (some of which would be extracellular polysaccharide) and a small (eight-42 per cent) increase in D for NaF. It was concluded that, contrary to much speculation, extracellular polysaccharides do not form a barrier to diffusion but instead lead to slightly faster rates of diffusion in plaque.

Finally, the effect of one month of daily mouthrinsing with 0.2 per cent NaF on plaque acidogenicity and plaque fluoride concentration was investigated for a group of 20 subjects. Plaque

acidogenicity was assessed by measuring plaque pH in vitro following fermentation with sucrose in situ (Stephan curve). Plaque fluoride concentration increased on average 12-fold during the rinsing period (plaque was sampled between eight and 12 hours after rinsing) and there was also an average increase (not statistically significant) of 0.12 units in the pH minimum. For 13 subjects whose pH minima prior to fluoride rinsing were less than 5.7 units, there was a statistically significant ($p < 0.01$) average increase of 0.22 units in the pH minimum with fluoride rinsing. There was a large reduction in plaque acid production when 0.2 per cent NaF was included for four subjects in the sucrose rinse used to induce the Stephan curve. Although the major mode of action of fluoride in reducing dental caries is by lowering enamel solubility and promoting remineralisation, this may be supplemented by reduced acid production in plaque in the presence of fluoride.

CHAPTER 1

INTRODUCTION

1.1 AIMS

The principal aim of the work reported in this thesis was to measure rates of diffusion in dental plaque, a tenacious deposit which forms on tooth surfaces and which comprises bacteria, their extracellular products and polymers of salivary origin. The results will be discussed in relation to the formation of dental caries, the localised dissolution of tooth enamel by acid derived from microbial degradation of dietary carbohydrates, and also to caries prevention, particularly by the use of fluoride.

In this introduction, the diffusion process will be defined and then the prevalence of caries, the theories of caries formation, the relevant properties of dental plaque, the importance of diffusion to caries and the mechanisms of action of fluoride in preventing caries will all be outlined.

1.2 DIFFUSION

Diffusion is a fundamental, irreversible process by which a difference of concentration is reduced by a spontaneous flow of matter (Robinson and Stokes, 1965, p. 45). This transfer of matter arises from the random motion of individual molecules and is distinct from mixing caused by convection or bulk motion

(e.g. stirring) of the system. By direct analogy to the theories of heat conduction, Fick, in 1855, formulated some mathematical expressions to describe diffusion.

The diffusion coefficient, D , is defined by the following equation, expressed here in the one-dimensional form, known as Fick's first law of diffusion (Crank, 1956; Jost, 1960):-

$$F = -D \frac{\partial c}{\partial x} \quad \text{Equation 1.1}$$

where F is the flux or rate of flow of substance by diffusion through unit area of a plane, c is the concentration of the diffusing substance, x is the distance coordinate measured perpendicular to the plane and therefore $\frac{\partial c}{\partial x}$ is the gradient of concentration across the plane. The negative sign in Equation 1.1 indicates that the direction of flow is opposite to that of increasing concentration. Equation 1.1 can be expressed in another form, known as Fick's second law of diffusion. For diffusion in one dimension only:-

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \quad \text{Equation 1.2}$$

where t is time and therefore $\frac{\partial c}{\partial t}$ is the rate of change of concentration with time. Substitution of the value of the diffusion coefficient and the appropriate boundary conditions into Fick's laws enable the rate of flow of substance by diffusion to be calculated.

It must be emphasised that Fick's laws assume that diffusion occurs in an isotropic medium and also that there are no irreversible chemical or metabolic interactions between the diffusing substance and the medium which either change the chemical

form of the diffusing substance or prevent it from further diffusion. If such interactions do occur, further terms must be introduced into Fick's laws to describe the process which should more correctly be called penetration or diffusion with reaction.

1.3 PREVALENCE OF DENTAL CARIES

Dental caries, or tooth decay, is the most common disease in civilised communities in which refined carbohydrates form a substantial part of the diet. In such communities, almost the entire population is affected to some extent by caries e.g. Keene et al. (1971) reported that between 1960 and 1969, only 0.2 per cent of 565,489 naval recruits in Illinois, USA, were caries-free and of 1,719 Danish army recruits in 1972, only 0.17 per cent were caries-free (Antoft, Gadegaard and Lind, 1974). In Scotland in 1972, 44 per cent of adults aged 16 years and over had no remaining natural teeth (Todd and Whitworth, 1974) and dental caries would be the major cause of tooth loss in the juveniles and young adults. A similar survey (Todd and Walker, 1980) showed that in the 10 years from 1968, the standard of dental health in England and Wales improved significantly but that in 1978, 29 per cent of the adult population were edentulous and the partially dentate adults had on average 13.2 caries-free and untreated teeth, 8.1 treated but otherwise sound teeth, 1.9 actively decayed teeth and 8.8 were missing. Thus, despite an upsurge over the last 30 or so years in the amount of research conducted into dental caries, a great deal has yet to be accomplished in the prevention of dental caries on a community basis.

1.4 THEORIES OF CARIES FORMATION

There are three main theories on the cause of dental caries (for reviews, see Kleinberg, 1977; Jenkins, 1978, pp. 414-465). The first, and most widely accepted theory, is the acid decalcification theory. This theory arose from the experiments of Miller (1890) who showed that when teeth were incubated with saliva and carbohydrate, acid was produced and some of the calcium and phosphate of the teeth dissolved.

The other two theories of caries formation were conceived after it was discovered that about one per cent of the enamel by weight was organic material (mainly protein and lipid). The proteolytic theory, proposed by Gottlieb(1947), suggests that the first step in caries formation is the proteolytic breakdown of the organic matrix of enamel by oral bacteria. This theory was modified (Schatz and Martin, 1962) to what is known as the proteolysis-chelation theory. This theory proposes that the products of proteolysis act as chelating agents which form complexes with calcium obtained by the breakdown of the mineral component of enamel. However, the extent of the proteolytic and chelative attack on enamel in vivo is unlikely to be severe enough to result in caries formation (Jenkins, 1978, pp. 427-429), although it may augment the process of enamel decalcification during acid attack, for which there is a vast amount of evidence.

It is now generally accepted that caries is formed by the dissolution of enamel by acid produced by the metabolism of dietary carbohydrates by plaque bacteria. This theory is particularly

supported by the experiments of Stephan (1940) who used fine antimony electrodes to measure the pH of intact plaque on anterior teeth. Within three minutes of rinsing the mouth with glucose solution, the pH of the plaque fell from an average of about 6.5 to about 5 and took up to 40 min to return to the original value. This 'Stephan curve' of plaque pH has been reproduced with a range of carbohydrates and foodstuffs by a number of workers, some using different techniques for pH measurement. The results show that following exposure to fermentable carbohydrate, the pH in plaque falls to values at which enamel dissolves in saliva e.g. the pH in carious cavities has been found to fall to as low as 4.4 (Caldwell and Bibby, 1958).

Jenkins (1965) has reviewed the experimental evidence available concerning the pH at which enamel dissolves in saliva. Above a certain pH value in the range 5.5 to 6.5, enamel was found not to dissolve in saliva, the exact value being dependent upon the composition of the saliva. This gave rise to the idea of a 'critical pH', below which the pH of saliva would have to fall before enamel would dissolve. This is explained by the low solubility of enamel at neutral pH and the saturation of saliva with calcium and phosphate. At lower pH values the enamel solubility increases and the saliva becomes unsaturated with calcium and phosphate, and so the enamel dissolves. Plaque contains much higher concentrations of calcium and phosphate and is a more powerful buffer than saliva (Strålfors, 1948). Thus the critical pH for dissolution of enamel in plaque would be expected to be lower than for saliva, but is not easily determined. Stephan (1944) and Englander, Carter and Fosdick (1956) observed that the Stephan curve for caries-active

subjects reached lower pH values than for caries-free subjects. From their results, Englander et al. deduced that the critical pH in plaque for enamel dissolution probably lies in the range 5.2 to 5.7. However, Tatevossian (1977) measured the solubility of enamel in plaque fluid over a range of pH values and has disputed the existence of a critical pH in plaque, and so this concept requires more thorough investigation.

The acid decalcification theory seems to be straightforward but in fact caries formation is a complex process. A range of factors are involved in determining the amount of acid produced in plaque, the pH value attained and the length of time for which the pH is lowered. Further, the process of enamel dissolution is complex. The early carious lesion is characterised by a loss of mineral from a site between 50 and 150 μm beneath the enamel surface (von der Fehr, 1966) and can be detected clinically as a "white spot" on the tooth. If decalcification is allowed to progress, the lesion becomes bigger, the relatively intact surface layer is lost and an open cavity is formed which in time may extend through the enamel to the dentine, eventually exposing the pulp and leading to pain, infection, necrosis, abscess formation and necessitating extraction of the tooth.

There is much experimental evidence, reviewed by Silverstone (1977) and by Moreno and Zahradnik (1979), to show that mineral can be deposited in enamel in vitro and in vivo in the presence of calcium and phosphate or saliva at near neutral pH, and carious lesions at the "white spot" stage can be repaired by

remineralisation. Thus it is now believed that caries progresses by alternate demineralisation, when the plaque pH falls after exposure to dietary carbohydrates, and partial remineralisation when the plaque pH rises again. Some of these factors involved in caries formation are discussed in more detail in the next two sections.

1.5 STRUCTURE AND PROPERTIES OF DENTAL PLAQUE

Dental plaque is a deposit of bacteria in an organic matrix which accumulates on the enamel surface if the teeth are not cleaned, and is not removed by rinsing or a water spray. Bacterial cells occupy about 70 per cent of the plaque by volume. The remaining 30 per cent is occupied by a matrix of selected salivary proteins (perhaps modified by bacterial enzymes), polysaccharide of bacterial origin and water.

After tooth cleaning, the first event in the reformation of plaque is the deposition of salivary glycoprotein to give a cell-free organic layer known as acquired pellicle (Dawes, Jenkins and Tonge, 1963; Meckel, 1965). The pellicle is rapidly colonised by salivary bacteria to form dental plaque, which increases in thickness over a period of days. The thickness of plaque layers is not well documented but has been estimated to range from five to 200 μm on smooth tooth surfaces (Meckel, 1965; Saxton and Critchley, 1970) and may be thicker in fissures and interproximal areas, whereas pellicle is only one to 10 μm thick (Meckel, 1965). Typically 10 to 20 mg of plaque (wet weight) will accumulate on the teeth of an individual in 24 h in the absence of tooth cleaning. Gibbons et al. (1964) reported

that plaque, of unknown age, contained about 250 million bacteria per mg wet weight, of which about one third were cultivable. The predominant cultivable microorganisms were as follows: facultative streptococci, 27%; facultative diphtheroides, 23%; anaerobic diphtheroides, 18%; peptostreptococci, 13%; Veillonella, 6%; Bacteroides, 4%; fusobacteria, 4%; Neisseria, 3%; vibrio, 2%. A range of other bacteria, such as lactobacilli, may also have been present but constituted less than 1% of the total microflora.

The microbial composition of plaque has been shown to change with the age of the plaque (Ritz, 1967; 1969). Aerobic cocci and rods were found to predominate in plaque accumulated for one day, with streptococci, Neisseria and Nocardia present in greatest numbers. The plaque thickness increased during a period of nine days accumulation and there was an increase in the proportion of anaerobic bacteria such as Veillonella and Fusobacterium and a corresponding decrease in the proportions of aerobes such as Neisseria and Nocardia. Together with this shift in predominance from aerobic to anaerobic bacteria, the plaque structure becomes more ordered as the plaque ages. The cocci and rods of 'young' plaque show little structural arrangement but in plaque accumulated for several days, about 50 per cent of the microorganisms appear to be present in a filamentous form. These are largely arranged perpendicular to the enamel surface with chains of cocci frequently to be found in between the filaments (McDougall, 1963; Newman, 1973). Plaque which accumulated in tooth fissures during a seven day period was reported by Theilade, Fejerskov and Hørsted (1976) to consist mainly of cocci and rods with some food debris but with few filaments,

and as such, differed from the plaque which would accumulate on smooth tooth surfaces in the same time interval.

The water content of plaque is 82 per cent by weight: 50 per cent is associated with the cells and 32 per cent with the matrix (Edgar and Tatevossian, 1971). Of the dry plaque material, 40 to 50 per cent by weight is protein, 13 to 17 per cent is carbohydrate and 10 to 14 per cent is lipid (Jenkins, 1978, p. 372). Plaque also contains a variety of minerals, including potassium, sodium, calcium, phosphate, magnesium and fluoride, which form about 10 per cent of the plaque dry weight. The fluoride concentration in plaque varies considerably and has been reported to range from about one to more than 100 parts per million (ppm) of plaque wet weight (see Chapter 9, this thesis).

Most of the plaque bacteria can produce acid by metabolising carbohydrates, particularly sucrose and glucose. Plaque acid production in vivo is most frequently monitored by measuring the Stephan curve of plaque pH. However, the presence of acid in plaque has been measured directly by Geddes (1975) and Gilmour et al. (1976), using the technique of gas liquid chromatography. Lactic, acetic, propionic, formic and butyric acids were shown to be present in plaque in concentrations of the order of 10^{-5} mmoles/mg plaque wet weight. Lactic acid was the principal acid produced on incubation of plaque with sucrose in vivo. More recent studies, using the technique of isotachopheresis (Geddes, personal communication, 1980), have shown that plaque also contains small amounts of pyruvic and succinic acids.

In addition to acids, three main groups of polysaccharides are formed by the metabolism of dietary carbohydrates by plaque bacteria (for review, see Newbrun, 1976; Hamilton, 1976). The enzymes known as glucosyltransferases from Streptococcus mutans, Streptococcus sanguis and other bacteria, polymerise the glucose moiety of sucrose to form extracellular glucans. These long chain polymers are gelatinous in appearance, form part of the insoluble matrix of plaque, and are only slowly degraded. Glucans are also important to the adherence of bacteria to each other and to the enamel surface. Fructosyltransferases, from bacteria such as Streptococcus mutans, Streptococcus salivarius and Actinomyces viscosus, polymerise the fructose moiety of sucrose to form extracellular fructans. Fructans are formed in much smaller quantities in plaque than are glucans, and are rapidly metabolised to form acid when the supply of sucrose is depleted. Finally, a wide range of plaque bacteria synthesise glycogen-like polysaccharides intracellularly from a variety of sugars, including sucrose, glucose and maltose. These intracellular polysaccharides are rapidly broken down to acid when supplies of other forms of sugar are depleted. Thus when plaque is exposed to sucrose, the pH will fall as a result of acid production by the plaque bacteria, but intra- and extracellular polysaccharides will also be produced. When the supply of sucrose has been exhausted, some of these polysaccharides will be broken down to form acid, thus prolonging the length of time for which the plaque pH is low.

Different bacteria have different acid-producing capacities and it is known that plaque from caries-active subjects reaches

lower pH values than plaque from caries-free subjects (Stephan, 1944; Englander, Carter and Fosdick, 1956). Consequently, much research has been directed at determining whether specific microorganisms are responsible for dental caries. Several studies have shown that the presence in plaque of Streptococcus mutans and Lactobacillus acidophilus is positively correlated with the presence of caries (for reviews, see Kleinberg, 1977 ; Jenkins, 1978, pp.431-434). However, it has yet to be established whether these bacteria are primarily responsible for causing caries or whether the proportions of these bacteria in the plaque increase in response to the formation of acidic conditions which are more favourable for the growth of these bacteria. Even in subjects with high caries activity, Streptococcus mutans and lactobacilli form a minor proportion of the total plaque flora, which suggests that the contribution of other bacteria to caries formation is important.

1.6 RELATION OF DIFFUSION TO CARIES

Various workers (Brown, 1974; Moreno and Zahradnik, 1974; Higuchi, 1974; Nancollas, 1974; Hopfenberg, 1974) have stated that a knowledge of rates of diffusion in dental plaque, acquired pellicle and enamel is essential for a full understanding of the caries process. Other workers (Holly and Gray, 1968; Higuchi et al., 1970; van Dijk, Borggreven and Driessens, 1979) have developed mathematical models based on diffusion processes, to describe caries formation. In this section, the role of diffusion in determining plaque pH and the rate of loss of mineral from enamel during acid attack will be discussed.

The first step involved in the production of acid in plaque is the diffusion of dietary carbohydrates from saliva into plaque. If diffusion is a relatively slow process then it could restrict the rate of acid production in plaque by limiting the availability of substrate. Some of the acid produced in plaque will diffuse through the pellicle into the enamel but acid will also be lost from plaque by diffusion into saliva and will then be removed from the mouth by swallowing. Flow of saliva over the plaque is important for another reason. Saliva contains large amounts of bicarbonate which may diffuse into plaque where it will buffer the acid and hence raise the plaque pH. Another factor which contributes to the raising of plaque pH is the ability of some plaque bacteria to produce alkali, mostly ammonia formation from urea but also the conversion of amino acids to amines by decarboxylation. Plaque pH will also be raised by the conversion of lactic acid to the weaker propionic and acetic acids by microorganisms such as Veillonella. Acid production in plaque will not cease until the supply of dietary carbohydrate is depleted and the intracellular and extracellular polysaccharides in the plaque have been broken down. Thus it is clear that the shape of the Stephan curve depends on many factors, at least three of which are concerned with diffusion i.e. diffusion of carbohydrate into plaque, clearance of acid from plaque by diffusion into saliva and diffusion of bicarbonate from saliva into plaque.

The fact that caries is a penetrating lesion which forms initially beneath the enamel surface, indicates that diffusion of acid in enamel plays an important role in caries formation. From

investigations of the formation of artificial carious lesions in enamel with lactic acid, Featherstone (1977) and Featherstone, Duncan and Cutress (1978; 1979) proposed a model based on diffusion phenomena to explain caries formation. The depth of penetration of the lesion was found to be proportional to the concentration of unionised lactic acid at the outer enamel surface. It was therefore suggested that caries formation was initiated by diffusion of undissociated lactic acid into the enamel via the fluid-filled inter-prismatic pores. As the lactic acid penetrates, some of it will dissociate and the hydrogen and lactate ions will react with the enamel. The resultant calcium and phosphate ions will then begin to diffuse back out of the enamel as a concentration gradient builds up. At the outer enamel surface, some of the calcium and phosphate ions will be precipitated because of the different ionic environment, thus maintaining a relatively intact surface enamel layer. Remineralisation will occur if the direction of the concentration gradients for calcium and phosphate are reversed i.e. by creating a high concentration of calcium and phosphate at the outer enamel surface.

Other physicochemical factors are also relevant to caries formation. The structure, composition and solubility of the enamel are particularly important, and the presence of fluoride or an acquired pellicle may reduce the solubility of the surface enamel. It is clear however, that caries initiation depends on diffusion of acids into enamel and the subsequent diffusion of calcium and phosphate from enamel.

1.7 PREVENTION OF CARIES WITH FLUORIDE

There is an abundance of studies which show that fluoride, administered in a variety of chemical and physical forms, is clinically effective in preventing caries. It is beyond the scope of this thesis to review the extent of caries reduction by each of the fluoride treatments. However, it should be noted that fluoride can be incorporated into teeth either systemically (if administered during the period of tooth development, prior to tooth eruption) or from topical application of fluoride to erupted teeth. Fluoride is often administered for systemic effect in domestic water supplies, salt, milk or in tablet form, and can be applied for topical effect in mouth-rinses, gels, varnishes, chewing-gum, toothpastes and tablets (Geddes, Jenkins and Stephen, 1973).

The literature concerning the mechanisms by which fluoride exerts its cariostatic effect is extensive and has frequently been the subject of review articles (Hardwick, 1963; Jenkins, 1967; Levine, 1976; Dreyer, 1979). It has long been established that tooth enamel when treated with fluoride becomes less soluble in acid. This is generally accepted as being due to the formation of fluorapatite and possibly other calcium-phosphate-fluoride complexes less soluble than the hydroxyapatite of tooth enamel. Recently it has been suggested that this reduction in enamel solubility is too small to fully account for the caries preventive action of fluoride (Brown, Gregory and Chow, 1977). Caries formation is generally accepted to be the result of a balance between enamel dissolution when the pH of plaque falls, and remineralisation when the plaque pH rises again. Fluoride has

been shown to favour the reprecipitation of calcium and phosphate in enamel, with fluorapatite being formed in preference to more soluble crystalline forms of calcium phosphate. Fluoride also promotes the formation of larger crystals of apatite with fewer imperfections, more resistant to acid attack. Thus, part of the cariostatic action of fluoride seems to arise from an ability to repair the damage to enamel caused by the initial acid attack. Several studies have shown that fluoride with saliva or calcium and phosphate can remineralise early carious lesions in vitro (Silverstone, 1977) and also in vivo (von der Fehr, Løe and Theilade, 1970; Levine, 1975; Edgar et al., 1978a). Although the main cariostatic mechanisms of fluoride seem to be due to the effects on enamel solubility and remineralisation, it is also known that fluoride is a powerful enzyme inhibitor. There is some evidence (to be reviewed in Chapter 9) that fluoride can reduce the acidogenicity of dental plaque by inhibiting acid production by plaque bacteria.

It should be noted that diffusion may be important in determining the effectiveness of topically applied fluoride preparations, because fluoride must diffuse through any layers of plaque or pellicle present on the tooth surface before reacting with the enamel.

The following chapter is a review of the literature on diffusion in dental tissues - enamel, pellicle and plaque.

CHAPTER 2

REVIEW OF THE LITERATURE ON DIFFUSION IN DENTAL TISSUES

2.1 DIFFUSION IN DENTAL ENAMEL

Several workers, using a variety of techniques, have attempted to quantify rates of diffusion in enamel. These studies yield different types of information about enamel permeability and should be interpreted with caution. Diffusion is a process of passive or non-interactive transport, described by Fick's laws of diffusion. Apparent diffusion coefficients have been calculated from studies of the penetration of ions into enamel. This process is the combined effect of diffusion and chemical interaction between the ions and the enamel, and such diffusion coefficients are of limited value as they cannot be applied to experimental conditions with different amounts of chemical reaction.

Fremlin, Hardwick and Mathieson (1959) showed that ^{18}F and ^{24}Na were able to penetrate enamel. The authors warned that in studies of uptake of radiolabelled tracers by enamel, three types of reaction may occur. Firstly, there may be uptake of the isotope on the surface of the enamel by hetero-ionic exchange e.g. ^{18}F exchanging with hydroxyl ions. Secondly, there may be surface uptake due to iso-ionic exchange e.g. ^{18}F exchanging with stable fluoride already bound within enamel. Thirdly, there may be penetration of the tracers into the enamel accompanied by hetero- and iso-ionic exchange. The uptake of ^{18}F by enamel was shown to be

preferentially by iso-ionic exchange, so this technique cannot be used to measure the uptake of stable fluoride by enamel. More recently, Tetteh (1974) showed that the uptake of ^{24}Na , ^{32}P , ^{45}Ca and ^{89}Sr by enamel was by iso- and hetero-ionic exchange. For ^{32}P uptake there was a strong preference for iso-ionic exchange and for ^{45}Ca uptake there was a strong preference for hetero-ionic exchange. The uptake of ^{24}Na and ^{89}Sr showed slight preference for iso-ionic exchange.

The uptake of ^{18}F from aqueous solutions and from dentifrices by enamel was investigated by Duckworth and Braden (1967). For a range of experimental conditions, the amount of ^{18}F taken up was proportional to the square root of the application time, indicating that the rate-controlling process was one of diffusion, and the exchange reaction was shown to be predominantly iso-ionic. A mathematical model, based on diffusion and simultaneous chemical reaction, was constructed to describe the uptake. Uptake of ^{24}Na by enamel was subsequently shown to be a simple diffusion process with no significant amount of chemical reaction (Braden, Duckworth and Joyston-Bechal, 1971). At 25°C the diffusion coefficient of ^{24}Na in enamel was about $2 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$. Measurements of the diffusion coefficient of ^{24}Na at various temperatures gave a value for the activation energy for diffusion of 5 kcal per mole, indicating that diffusion occurs through a predominantly aqueous phase. This was confirmed (Joyston-Bechal, Duckworth and Braden, 1971) by measuring the uptake of ^{18}F and ^{24}Na by pieces of enamel subjected to various dehydration treatments. The uptake remained diffusion-controlled but the total amount taken up decreased as the enamel became less hydrated. It was further shown (Joyston-Bechal, Duckworth

and Braden, 1973) that significantly more ^{18}F was taken up in 5 min by enamel from an acidulated phosphate fluoride (APF) solution than from a neutral NaF solution. The initial pattern of uptake from APF was not diffusion controlled but arose from surface deposition caused by the acidic conditions.

The uptake of stable fluoride by enamel from APF solutions was measured by Stearns (1970), and a penetration profile was constructed i.e. a plot of fluoride concentration against depth into the enamel. A mathematical model based on diffusion and simultaneous chemical reaction was constructed to describe the uptake process, assuming that all the fluoride in the enamel was present as fluorapatite and that there was no CaF_2 deposition. Using this model, apparent diffusion coefficients of fluoride in enamel were calculated from the penetration profiles (Stearns, 1971). The apparent diffusion coefficient was $1.4 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ at 22°C , $1.3 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ at 29°C and $1.8 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$ at 37°C i.e. the apparent rate of diffusion decreased as the temperature was raised. This is contrary to classical diffusion theory and occurs because the diffusion coefficient has been used to describe penetration, which is dependent upon chemical reaction and diffusion. The exchange of fluoride for hydroxyl ions in enamel is an exothermic chemical reaction, which therefore is not favoured at high temperatures and so the uptake of fluoride is less at higher temperatures. To assign values of diffusion coefficient to this process is incorrect and misleading as these values cannot be applied to different experimental conditions.

The penetration of ^{45}Ca and $^{32}\text{PO}_4$ ions into single crystals of fluorapatite at temperatures ranging from 1000 to 1800 $^{\circ}\text{K}$, was investigated by den Hartog, Welch and Royce (1972). By constructing penetration profiles, it was shown that calcium penetrated faster than phosphate by two orders of magnitude. Extrapolation of the experimental data yielded diffusion coefficients of the order of $10^{-45} \text{ cm}^2 \text{ s}^{-1}$ at body temperatures, which implies penetration depths of the order of 1\AA in 10^{20} years. This suggests that in tooth enamel there would be no penetration of ions into crystallites of hydroxyapatite, which are similar to fluorapatite in structure.

Waters (1970) constructed a diaphragm diffusion cell, in which a thin slice of enamel was mounted to form a window between two fluid-filled chambers. Diffusion coefficients could be measured by monitoring the passage of substances from one chamber through the enamel into the other chamber, although no values were reported. Waters (1971; 1975) measured electrical potentials across enamel sections mounted in the cell and showed that enamel behaved as an ion exchange membrane with characteristics dependent upon the ionic composition of the surrounding fluid. In solutions of KCl , KH_2PO_4 , K_2HPO_4 or K_3PO_4 , the enamel behaved as a cation exchange membrane, but behaved as an anion exchange membrane in solutions of CaCl_2 .

Using a diaphragm diffusion cell technique, Moreno and Burke (1974) measured the diffusion coefficient of tritiated water in enamel at 37 $^{\circ}\text{C}$ to be $1.16 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$. In a subsequent study (Burke and Moreno, 1975) the diffusion coefficient was reported to

range from 0.7 to $3.0 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ and was dependent upon the source of the enamel sample. Diffusion through canine enamel was faster than through incisor enamel. From measurements of diffusion at different temperatures, the activation energy for diffusion was calculated to be $4.46 \text{ kcal mole}^{-1}$. This is similar to the value reported by Braden, Duckworth and Joyston-Bechal (1971) and is consistent with diffusion in a predominantly aqueous medium. It was considered that diffusion in enamel was mainly through inter-prismatic pores, containing water and organic material, although there would be a small amount of penetration into the narrower inter-crystallite pores. This experiment is relatively free from the effects of chemical interaction because only freely-diffusible tracer was detected.

The diaphragm diffusion cell technique was also used by Borggreven, van Dijk and Driessens (1977) to measure the simultaneous diffusion of $^{86}\text{Rb}^+$, $^{36}\text{Cl}^-$, ^{14}C -glycerol and ^3H -sorbitol through thin slices of bovine enamel at 4°C . Diffusion coefficients varied by up to two orders of magnitude, depending on the slice of enamel studied. Diffusion coefficients ranged from 1.2×10^{-7} to $3 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ for $^{86}\text{Rb}^+$, from 8×10^{-8} to $2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ for $^{36}\text{Cl}^-$, from 4.5×10^{-8} to $8 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ for ^{14}C -glycerol and from 2.5×10^{-8} to $4 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ for ^3H -sorbitol. The enamel appeared to be ion selective, with cations more mobile than anions, and a molecular sieve effect was observed for glycerol and sorbitol i.e. because of their size, these molecules had difficulty penetrating the narrow pores in enamel. The results of this study are consistent with those of Burke and Moreno (1975).

Flim and Arends (1977a) studied the penetration of ^{45}Ca into bovine enamel. Two diffusion terms and an exponential term were used in an empirical mathematical equation to describe the complicated penetration profiles. A fast diffusion term (diffusion coefficient of $2.8 \times 10^{-12} \text{ cm}^2 \text{ s}^{-1}$) and a slower diffusion term (diffusion coefficient in the range 2×10^{-12} to $5 \times 10^{-14} \text{ cm}^2 \text{ s}^{-1}$) were suggested to correspond to inter- and intra-prismatic penetration respectively. From measurements of penetration at different temperatures, activation energies for diffusion were calculated to be about 0.7 and 1.3 eV (Flim and Arends, 1977b). The penetration profiles were discussed in relation to possible pathways for diffusion in enamel. Diffusion into single crystallites was regarded to be too slow (den Hartog, Welch and Royce, 1972). A rapid diffusion was associated with the presence of cracks in the enamel. There would also be diffusion through inter- and intra-prismatic pores, the former being faster because the pores are wider. The same experimental technique was employed by de Rooij and Arends (1980) to study the penetration of ^{32}P as KH_2PO_4 into bovine enamel. Two diffusion terms were required to describe the penetration profile, with a fast diffusion coefficient of about $2 \times 10^{-12} \text{ cm}^2 \text{ s}^{-1}$ and a slow diffusion coefficient in the range 10^{-17} to $10^{-21} \text{ cm}^2 \text{ s}^{-1}$.

Featherstone, Duncan and Cutress (1978; 1979) studied the formation of artificial subsurface carious lesions in enamel in vitro. From measurements of the rate of movement of the boundaries of the lesions, apparent diffusion coefficients for acids and calcium phosphate products of enamel dissolution were estimated to be of the order of $10^{-10} \text{ cm}^2 \text{ s}^{-1}$.

In summary, there have been two main approaches to measuring diffusion coefficients in enamel. The diaphragm diffusion cell technique eliminates many of the effects of chemical interaction. Diffusion coefficients measured by this technique are about 10^{-8} to 10^{-9} $\text{cm}^2 \text{s}^{-1}$ and probably reflect the fastest pathway for diffusion in enamel i.e. the inter-prismatic pores. Apparent diffusion coefficients have also been calculated from studies of penetration into enamel. This process is the combined effect of diffusion and chemical reaction and the apparent diffusion coefficients, ranging from 10^{-10} to 10^{-21} $\text{cm}^2 \text{s}^{-1}$, suggest much slower diffusion than do the diaphragm diffusion cell studies. These apparent diffusion coefficients should not be applied to other experimental conditions in which the amount of chemical reaction is different.

2.2 DIFFUSION IN PELLICLE

Pellicle occupies an important site in the mouth, acting as an interface between enamel and plaque or saliva. For some time it has been suggested that pellicle helps to protect enamel against acid dissolution (Dobbs, 1932a; Darling, 1943). These observations have been confirmed in recent years (Meckel, 1965; Zahradnik, Moreno and Burke, 1976; Zahradnik, Propas and Moreno, 1977). However, it has not been established whether this protection arises from pellicle acting as a diffusion barrier to prevent acids diffusing into enamel, or whether the pellicle somehow reduces the solubility of the enamel.

There is a scarcity of experimental measurements of diffusion in pellicle. In one of the few studies, Schüle (1961) used a diaphragm diffusion cell to monitor the rate of passage of lactic acid, glucose, calcium, phosphate and two dyes (congo red and trypan blue) through a layer of pellicle. The pellicle had been fixed for 5 hours in 96 per cent alcohol and then floated off the enamel in 2 per cent HCl. The calcium, phosphate, lactic acid and glucose all diffused through the pellicle but the high molecular weight dyes did not. The effect on pellicle permeability of the methods used to prepare the pellicle for study could not be assessed but it was concluded that pellicle did not protect enamel from acid dissolution by preventing the diffusion of small ions. Diffusion coefficients could easily have been calculated from this experiment but unfortunately this was not done, and the information required to enable an estimation to be made is not reported. Schüle (1963) also observed the presence of an intact pellicle on areas of enamel with subsurface demineralisation. This further suggests that pellicle is permeable to acids and to calcium and phosphate ions produced by enamel dissolution.

Moreno (1975) and Zahradnik, Moreno and Burke (1976) postulated that pellicle protects enamel not by restricting the rate of diffusion of all substances, but by acting as an ion-selective membrane. Using a diaphragm diffusion cell (Moreno and Burke, 1974) a study was made of the rate of passage of radio-labelled ions through a disc of compressed hydroxyapatite powder. Experiments were repeated following immersion of the disc in whole saliva for varying lengths of time. The artificial pellicle which formed on

the disc exhibited ion-selective properties, but only after a minimum of seven days growth. The presence of the pellicle retarded the rate of diffusion of $^3\text{H}_2\text{O}$ by a small amount (10 per cent) but there was a 25 per cent reduction in the rate of diffusion of $^{14}\text{CNS}^-$ and a 50 per cent reduction for $^{35}\text{SO}_4^{2-}$. Unfortunately the thickness of the pellicles was not determined and diffusion coefficients could not be calculated. The experiment has to be extended to include a wider range of cations as well as anions before the degree of ion-selectivity exhibited by pellicle can be adequately assessed. The importance of this effect can only be evaluated when the diffusion coefficients are known together with the total time required for diffusion through pellicle in vivo.

There are three further studies which indicate that the diffusion-restricting properties of pellicle may not be of clinical importance. Tinanoff, Wei and Parkins (1974; 1975) and Joyston-Bechal, Duckworth and Braden (1976) showed that a coating of pellicle did not significantly reduce the amount of fluoride taken up by enamel from sodium fluoride or acidulated phosphate fluoride solutions in vitro or in vivo.

In summary, there is some evidence which shows that pellicle does not significantly restrict the diffusion of small molecules and ions although it exhibits some degree of permselectivity. The caries-preventive properties of pellicle are more likely to arise from a reduction of enamel solubility than from a restriction of diffusion.

2.3 DIFFUSION IN DENTAL PLAQUE

Diffusion in dental plaque has been widely stated to be of importance in the initiation and progression of dental caries. However, there is a lack of quantitative measurements of rates of diffusion in plaque. The literature relevant to diffusion in plaque is reviewed in two parts. The first part establishes the importance of diffusion in plaque by reviewing the published qualitative assessments of diffusion rates. The second part deals with the attempts to quantify the rates of diffusion.

2.3.1. Qualitative Assessments of Diffusion in Plaque

In an attempt to explain why sucrose seemed to be more cariogenic than starch, König and Grenby (1965) suggested that sucrose molecules could rapidly diffuse into plaque, resulting in the production of organic acid at the tooth surface. The larger starch molecules were thought to be unable to penetrate plaque, so that acids would only be formed at the outer surface of the plaque where they would be more readily washed away in saliva. This theory was extended when König (1966) considered that slimy extracellular polysaccharides would constitute a plaque matrix with the physico-chemical property of acting as a diffusion barrier, capable of trapping acids formed deep under the surface of the plaque.

This suggestion seemed to become accepted as fact in the dental literature despite the lack of experimental evidence. Critchley et al. (1967) showed that both intra- and extra-cellular polysaccharides were synthesised in plaque from sucrose, whereas only intracellular polysaccharides were synthesised from glucose.

It was speculated that diffusion-limiting properties of extracellular polysaccharides might explain why sucrose is more cariogenic than glucose. In a review article on the cariogenicity of sucrose, Winter (1968) also propounded the concept that plaque extracellular polysaccharides would trap acid close to the enamel surface.

No explanation was offered to resolve the seemingly paradoxical situation of sugars being able to diffuse rapidly in plaque while acids diffused very slowly, until a paper by Kleinberg (1970). He speculated that fasting plaque might have a floccular structure - "a macromolecular arrangement with spaces between the aggregating substances". This would facilitate rapid uptake of sugars, resulting in a rapid production of extracellular polysaccharides and therefore a more gel-like plaque. It was considered that diffusion of acids in this gelatinous medium would be relatively slow.

A study by Guggenheim, Regolati and Mühlemann (1972) showed that a group of rats fed a diet containing mutanase, an enzyme capable of breaking down mutan (a form of polysaccharide) in dental plaque, developed a lower caries incidence than a second group fed the same diet but without the mutanase. However, it was found that the extent of the deposits of plaque developing on the smooth surfaces of the teeth was the same in both rat groups. The authors explained their results on the basis that mutan controlled the rates of diffusion in plaque. It was suggested that plaque cariogenicity was governed by its microbial composition and by its diffusion characteristics, and could not be adequately assessed by measuring

the extent or the thickness of the plaque. Mutan would indeed be an important diffusion-regulating component of plaque if its effect was greater than that of varying the thickness of the plaque, because the time required to diffuse a certain distance is proportional to that distance to the second power.

These concepts were perpetuated by other workers. In a review article, Mandel (1974) stated that the cariogenicity of plaque arises partly from its structural nature which would favour retention of acids at the enamel surface and restrict the diffusion of neutralising agents into the plaque. Saxton (1975) also stated that plaque acted as a diffusion barrier to produce these effects. Cole (1977) stated that insoluble glucans formed a diffusion barrier in dental plaque.

In a review article, Bowen, (1976) suggested that the most deleterious property of plaque extracellular polysaccharides was an ability to restrict the diffusion of neutralising agents through plaque. The electrostatic charge on the polysaccharides was considered to be sufficient to severely retard the diffusion of small ions whereas sugars could diffuse rapidly. This concept was repeated in a second article (Bowen, 1978) in which a paper by Rorem (1955) was cited as substantiation. Rorem revealed that extracellular polysaccharides had the capacity to bind large amounts of $^{86}\text{Rb}^+$ and $^{32}\text{PO}_4^{3-}$ ions. However, information on rates of diffusion in plaque cannot be derived from this study as it merely suggests that, as was found for enamel, some of the ions may become bound within the diffusion medium as the diffusion proceeds. This

should apply to acids and products of dissolution as well as neutralising agents.

Melvaer, Helgeland and Røilla(1974) showed the presence of lipoteichoic acids (polymers of glycerol and phosphate) associated with polysaccharides on the cell walls of Streptococcus mutans and Streptococcus sanguis incubated with sucrose. Negatively charged phosphate groups on the lipoteichoic acids were shown to be of importance to the adherence of cells to hydroxyapatite (Ciardi et al., 1977). It was also speculated that calcium ions binding to lipoteichoic acids associated with extracellular polysaccharides in the plaque matrix might act as a diffusion barrier, preventing further diffusion of positively charged ions although having little effect on diffusion of uncharged molecules.

The importance of diffusion in plaque to the caries process is apparent from these papers. The main question arising is: to what extent do diffusion processes control the exposure of enamel to acid attack? The following section reviews the studies designed to measure some of the quantitative aspects of diffusion in plaque.

2.3.2 Quantitative Measurements of Diffusion in Plaque

The first reported attempt to measure diffusion in dental plaque was made by Dobbs (1932b). A crude diaphragm diffusion cell was constructed and used to measure the amounts of KOH, Na_2HPO_4 and lactic acid capable of passing through layers of plaque which were removed from extracted teeth by immersion in 5 per cent HCl.

Lactic acid was found to pass through plaque more quickly than KOH and Na_2HPO_4 . However, diffusion coefficients could not be calculated without modifications to the experimental design. The effect of treatment with 5 per cent HCl on the plaque structure and permeability could not be assessed and so caution should be exercised in applying these results to plaque in situ.

Muntz and Miller (1943) investigated the ability of high molecular weight detergents such as Zephiran (alkyl dimethyl benzyl ammonium chloride) to inhibit lactic acid production from exogenous glucose in plaque sediment. The detergents were not able to diffuse into the sediment quickly enough to completely inhibit acid production. When the thickness of the sediment was increased from 0.5 to 0.75 and then to 1.0 mm, the degree of inhibition was found to decrease. This effect demonstrates that the detergents diffused into the sediment more slowly than glucose.

Nevin and Walsh (1951) and Nevin (1954) investigated the effect of some physico-chemical factors on the rate of clearance of acid from interproximal sites between teeth. Using glass models and monitoring pH, the shape of the site (its width and depth), the type of acid, the buffering capacity of the surrounding fluid, stirring or agitation, and blockage by solid material were all shown to be of importance to the rate of clearance of acid.

A diffusion cell was designed by Forscher and Fosdick (1954) to monitor the passage of hydrogen ions from a sucrose solution through a membrane permeable to hydrogen ions but not to

sucrose. The rate of diffusion of hydrogen ions decreased as the sucrose concentration was increased from 0 to 1.5 M. This is the opposite effect to that expected from osmosis but is consistent with the general observations that rates of diffusion are inversely proportional to the viscosity of the medium (see Appendix). In a second study Fosdick and Forscher (1954) reported similar results for the diffusion of calcium and phosphate ions.

Manly (1958) undertook a study which was more extensive but very similar in design to that of Muntz and Miller (1943). Manly measured the ability of 62 different compounds to inhibit glycolysis within layers of salivary sediment 0.2, 0.5 and 0.9 mm in thickness. The degree of inhibition by the compounds varied widely, but in each case was inversely related to the thickness of the sediment. It can be concluded that these compounds, almost all of which were of high molecular weight, diffuse into sediment more slowly than glucose.

Huh, Blackwell and Fosdick (1959) measured the penetration and simultaneous metabolism of glucose into a 1 mm layer of salivary bacterial sediment. Penetration profiles were constructed to show the concentrations of glucose and lactic acid against depth into the layer for various initial glucose concentrations and equilibration times. The authors concluded that the sediment possessed a resistance to the penetration of glucose. However, the simultaneous diffusion and metabolism does not permit diffusion coefficients to be calculated to substantiate this claim. It must also be emphasised that salivary sediment differs from plaque in its

proportional distribution of bacteria (Socransky and Manganiello, 1971) and shows no structural organisation.

Gilders (1961) proposed the theory that a factor essential to caries formation was the presence of an inert, impenetrable layer of food debris over acidogenic plaque to prevent saliva exercising a neutralising effect. An experiment was designed to test whether neutralising agents could diffuse from a pool of saliva through a cellulose fibre barrier and prevent the metabolism of sucrose in a sample of plaque. The experimental results showed large variations and no information on diffusion in plaque can be concluded from this study.

A diaphragm diffusion cell was constructed by Nolte and Arnim (1964) with a disc of plaque forming a window between two fluid-filled chambers. When glucose or sucrose was added to one chamber, the pH in both chambers dropped rapidly. It was concluded that sugars rapidly penetrated the plaque where they were metabolised to form acids. This technique could be adapted to quantify rates of diffusion in plaque by stirring the fluid in each chamber, measuring concentration instead of pH and preventing metabolic activity within the plaque.

The rate of release of ^{32}P and ^{45}Ca from a block of enamel into broth solutions under acidic conditions was found to be restricted by the presence of a bacterial coating on the enamel (Bowden, Nash and Spiers, 1973). The bacterial coating was produced by immersing the enamel in a broth culture of

Streptococcus sanguis with sucrose as the carbohydrate source. The resultant deposit of bacteria and extracellular polysaccharide matrix was acknowledged by the authors to bear little relationship to human dental plaque. When the deposits were killed by exposure to 0.2 per cent formalin there was a further decrease in the clearance rates. The authors explain the results as being largely due to restricted diffusion through the deposits. However, the experiment involves several processes - diffusion, acid production, enamel solubility, reprecipitation, binding to organic material - and it is difficult to extract quantitative information on diffusion from this system.

Hojo, Higuchi and Araya (1976) reported an experiment which they claimed provided evidence in support of the belief that glucan behaved as a diffusion barrier. They used two strains of Streptococcus mutans PK1, a mutant and a wild strain, which produced relatively small and relatively large amounts of insoluble glucan respectively. Deposits of each were grown on glass pH electrodes immersed in broth cultures containing sucrose. The pH measured with the electrode coated with the mutant strain closely matched the pH of the medium during a 40 hour incubation. When the experiment was repeated with the wild strain, the pH recorded by the coated electrode fell below the pH of the medium. However, the authors did not investigate the rates of acid production from sucrose or the types of acids produced for each of the strains, and the thickness of the layers was not reported. It has therefore not been established that the different pH values were a result of a restriction of diffusion.

A more comprehensive study of the same type was recently performed by Johnson and Hillman (1980). A pH electrode coated with a deposit of S. mutans strain BHT-2 recorded pH 4.6 in a sucrose-containing medium of pH 5.4. A second electrode with a much more copious deposit of an LDH-deficient strain of S. mutans (JH 145) recorded pH 5.4 in a medium of pH 5.8. The principal acidic products of metabolism were lactic acid and formic acid for the parent (BHT-2) and mutant (JH 145) strains respectively. Organic acids were shown to penetrate at similar rates in each of the bacterial deposits but formic acid penetrated faster than lactic acid. Also, the mutant strain ceased acid production at a higher pH and produced less acid per mole of carbohydrate than the parent strain. Thus the difference in pH values recorded by the coated electrodes was explained by factors other than the presence of a diffusion barrier.

Three studies, carried out during the same period as the work reported in this thesis, come closer to providing quantitative measurements of diffusion in plaque. Melsen *et al.* (1979) studied the penetration of ^{45}Ca (as CaCl_2), ^{32}P (as Na_2HPO_4), ^3H -glucose and ^3H -glucosamine into columns of dental plaque at low temperatures. From penetration profiles it was seen that ^{45}Ca penetrated in greater amounts and to greater depths than ^{32}P and ^3H -glucosamine, which in turn penetrated in greater amounts than ^3H -glucose. The authors conclude from this evidence that plaque may be permselective. This should be interpreted with caution, because the radiolabels may have been bound (by iso-ionic and hetero-ionic exchange) to the plaque constituents during the diffusion process.

Tatevossian (1979) used a variation of this technique to study the penetration of radiotracers into columns of dental plaque fluid and sediment. Radiotracers were applied to one end of the sample and apparent diffusion coefficients were calculated from penetration profiles. The fluid and sediment were obtained by centrifuging pooled plaque samples at 5000 g for 15 min. The apparent diffusion coefficients at 37°C of bicarbonate, acetate and lactate were about 3×10^{-6} and $1 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for plaque fluid and sediment respectively, and for sucrose was $5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for fluid and $0.9 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for sediment. Thus, the rate of penetration of ions into plaque fluid was about three-fold slower than into water, and into plaque sediment was about eight-fold slower than into water. Sucrose penetrated into water and plaque fluid at the same rate and six-fold more slowly into plaque sediment. These diffusion coefficients should be interpreted with caution because metabolic activity and binding of tracers to the constituents of the fluid or sediment may have interfered with the diffusion process. These effects may be particularly important at the low concentrations of tracers used by Tatevossian. It is also very difficult to apply these results measured for plaque fluid and sediment, to intact plaque.

In a subsequent study (Tatevossian and Newbrun, 1980) the rates of penetration of radio-labelled tracers into plaque sediment were ranked in the following order (fastest first): NaCl, NaH_2PO_4 , Na_2HPO_4 , NaHCO_3 , Na_2SO_4 , CaCl_2 . Calcium penetrated more slowly than phosphate, which is in contrast to the results of Melsen et al. (1979). Rates of diffusion in different media were

ranked as follows (fastest first): water, plaque fluid, submaxillary saliva, parotid saliva, plaque sediment.

Diffusion coefficients in plaque were measured more reliably by Dibdin (1979, and personal communication) using a diaphragm diffusion cell. The plaque samples were heated at 60°C for 30 min to prevent metabolic activity. The following values of diffusion coefficient at 35°C were reported: $2.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for lactate, acetate and propionate; $2.0 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for glucose and fructose, and $1.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for sucrose. These diffusion coefficients are five-fold smaller than the corresponding values for water. Plaque was only slightly permselective and acids diffused slightly faster below their pK_a 's. A criticism of this technique is that the heat treatment may affect the diffusion properties of the plaque, and there may be changes within the sample during the 5 h required for each experiment.

Finally, there are three studies which suggest that fluoride may diffuse rapidly through plaque. Kirkegaard et al. (1975) showed that a coating of 'artificial' plaque (bacterial deposits of Streptococcus sanguis or Streptococcus mutans grown in sucrose-containing broth) did not significantly reduce the amount of fluoride taken up by enamel from a two per cent sodium fluoride solution in vitro. Joyston-Bechal, Duckworth and Braden (1976) showed that a pellicle or plaque coating did not reduce the amount of ^{18}F taken up by enamel during a 4 min application of an acidulated phosphate fluoride solution or a sodium fluoride solution in vitro, and it was thought that the plaque and pellicle may result in a

greater amount of fluorapatite formation and less CaF_2 deposition. Bruun and Stoltze (1976) showed that a coating of plaque did not reduce the amount of fluoride taken up by enamel from an amine fluoride solution in vivo and seemed to enhance the fluoride uptake from a neutral NaF solution.

From this review of the literature it is seen that diffusion processes in dental tissues may play a critical part in caries initiation and prevention. However, the knowledge of rates of diffusion is scant, particularly for plaque and pellicle. One of the aims of this thesis is to make quantitative measurements of rates of diffusion in dental plaque.

CHAPTER 3

MEASUREMENT OF THE DIFFUSION COEFFICIENT OF 133 -XENON

IN HUMAN DENTAL PLAQUE

3.1 INTRODUCTION

The review of the literature on diffusion in dental plaque establishes the necessity of quantitative measurements of diffusion coefficients. Previous studies of diffusion in plaque have been unsatisfactory, mainly owing to a failure to separate the influences of chemical reaction and metabolism from the diffusion process. The aim of the experiments reported in this chapter was to measure diffusion coefficients in dental plaque from individual subjects, using a technique which allows measurements to be made with no metabolic or chemical interferences. In particular it was desired to determine whether or not dental plaque behaves as a diffusion barrier.

To circumvent the possible interference from metabolic or chemical activity, the technique used by Unsworth and Gillespie (1971) to measure diffusion coefficients of xenon and krypton in water and biological tissues, has been modified to study diffusion in plaque. The radioisotope ^{133}Xe , which emits γ -rays of energy 81 keV with a half-life of 5.3 days, is ideal for such a study. Xenon is chemically and metabolically inert and although it occurs naturally in the gaseous state it is soluble in water, where it is electrically neutral and non-polar. Comparison of the diffusion

coefficient of xenon in plaque with that of xenon in water, allows the diffusion characteristics of plaque to be assessed.

3.2 MATHEMATICAL THEORY

If xenon gas is allowed to dissolve into a thin layer of plaque of thickness L (cm), until it reaches a uniform concentration c_0 , and then the xenon gas from the surrounding medium is rapidly removed, the xenon will diffuse out of the plaque. The average xenon concentration $c(t)$, within the plaque at any subsequent time t (seconds), is given by the following expression (Jost, 1960, p.36):

$$c(t) = \frac{8 c_0}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \left[-D(2n+1)^2 \pi^2 t / 4L^2 \right]$$

Equation 3.1

where $n = 0, 1, 2, \dots$, and D is the diffusion coefficient of xenon in the plaque sample ($\text{cm}^2 \text{s}^{-1}$). After a time approximately equal to $0.1 L^2/D$, during which about 30 per cent of the dissolved xenon will have diffused out of the plaque, $c(t)$ is well approximated by the first exponential in the series:

$$c(t) = \frac{8 c_0}{\pi^2} \exp \left[-D\pi^2 t / 4L^2 \right]$$

Equation 3.2

This can be expressed in the form:

$$c(t) = c_1 \exp \left[-0.693 t / T_{\frac{1}{2}} \right]$$

Equation 3.3

which is analogous to the radioactive decay law, where c_1 is a normalising constant and $T_{\frac{1}{2}}$ is the clearance halving-time i.e. the time (in seconds) for the average xenon concentration within the plaque to decrease by a factor of two. From Equations 3.2 and 3.3,

it follows that:

$$D = \frac{0.2809 L^2}{T_{\frac{1}{2}}} \quad \text{Equation 3.4}$$

3.3 MATERIALS AND METHODS

3.3.1 Plaque Collection

Except where otherwise stated, the plaque used for the experiments reported in this thesis was collected from a group of young, partially dentate adult subjects who were members of staff of the Glasgow Dental Hospital and School and who had controlled, moderate rates of dental caries. As less than 30 mg of plaque were required for each experiment, separate measurements were made on plaque from individual subjects.

The plaque was removed from the teeth using a nickel microspatula, the edges of which had been ground and polished smooth. Plaque was taken from all available sound smooth surfaces, care being taken to avoid sites of carious lesions, restorations or deposits of calculus. All of the plaque samples were collected at least 1.5 hours after the subject's last consumption of food or drink. This corresponds to the 'resting plaque' referred to by Geddes (1974) and should minimise any complications due to rapid carbohydrate metabolism within the plaque.

Four subjects accumulated plaque for the study described in this chapter by refraining from tooth cleaning for periods of between one and four days. Subjects SM and RD produced plaque in

sufficient quantity in a 24 h period but subjects MM and HM accumulated plaque more slowly and so their plaque was collected after three or four days growth.

3.3.2 Xenon Preparation

The ^{133}Xe was purchased from the Radiochemical Centre, Amersham, England (Medical Product Catalogue, code no. XAS.21P). Ten mCi of ^{133}Xe were supplied dissolved in 1 ml of sterile isotonic saline inside a cartridge. This saline was drawn into a 2 ml Pressure-lok gas-tight syringe (Chromatography Services Ltd., 23 Old Chester Road, Wirral, Merseyside, England) along with 1 ml of air. The syringe was left for at least 24 h prior to the experiment for the ^{133}Xe to come out of solution into the air space. At equilibrium, approximately 90 per cent of the ^{133}Xe will be in the gaseous state.

3.3.3 Experimental Details

To hold the plaque samples, a set of three brass dishes was made. A well of diameter 8 mm was countersunk into each dish, to depths of approximately 0.35, 0.5 and 0.6 mm. The plaque was layered into one of the dishes to form a disc of plaque of uniform thickness (Fig. 3.1). The orientation of the plaque as it had developed in situ could not be preserved but care was taken to introduce as little disruption as possible and lumps of plaque were not dispersed. Histological sections of the samples were prepared (details to follow) for assessment of the plaque structure.

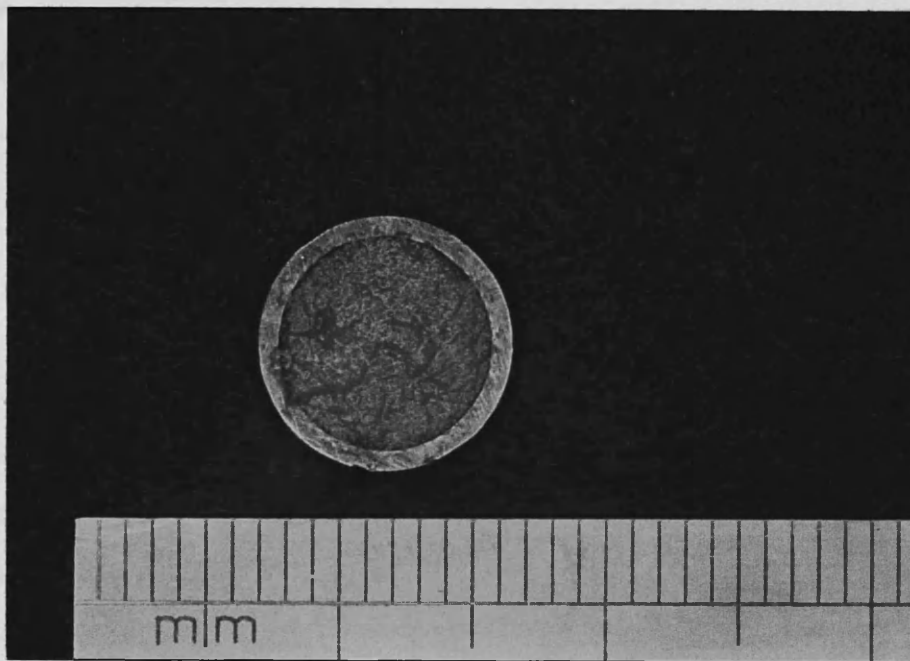


Fig. 3.1 Brass dish containing plaque sample for xenon diffusion experiment. Magnification x 3.6.

The dish was supported in an inverted position on a stainless-steel wire frame inside a brass diffusion chamber. The plaque was observed to remain in this position throughout the experiment. A gas-tight seal was achieved by compression of soft indium wire as the lid was screwed into position (Fig. 3.2). Inlet and outlet tubes, which allowed air to be pumped through the chamber, were sealed using mercury valves. Approximately 5 mCi of ^{133}Xe were injected into the chamber via a mercury-filled side arm at the outlet valve (Fig 3.3). All parts of this apparatus were contained in a perspex box, maintained at 37°C by a fan heater with thermostat control, and at high humidity. The total time required to remove the plaque from the mouth, layer it in the dish, seal it in the chamber and inject the ^{133}Xe was about 5 min.

Twenty minutes were allowed for the xenon to reach equilibrium within the plaque, after which, by lowering the mercury reservoir, the valves on either side of the chamber were opened and simultaneously an air pump was switched on, drawing humidified air through the chamber (internal volume 3 cm^3) at a rate of approximately $25\text{ cm}^3\text{ s}^{-1}$. The subsequent clearance of xenon from the plaque was monitored by detecting the 81 keV γ -rays from the ^{133}Xe using a sodium iodide scintillation counter, coupled via a pulse height discriminator to a digital rate-meter (EKCO Electronics Ltd., Essex, England, unit numbers M5401, M5010 and M5183A) and printer (Model D4-E, Kienzle Data Systems Ltd, London, England). The base of the diffusion chamber was made very thin (0.3 mm) to ensure efficient penetration by the γ -rays and a lead cover was placed above and around the chamber to reduce the background count rate. As the

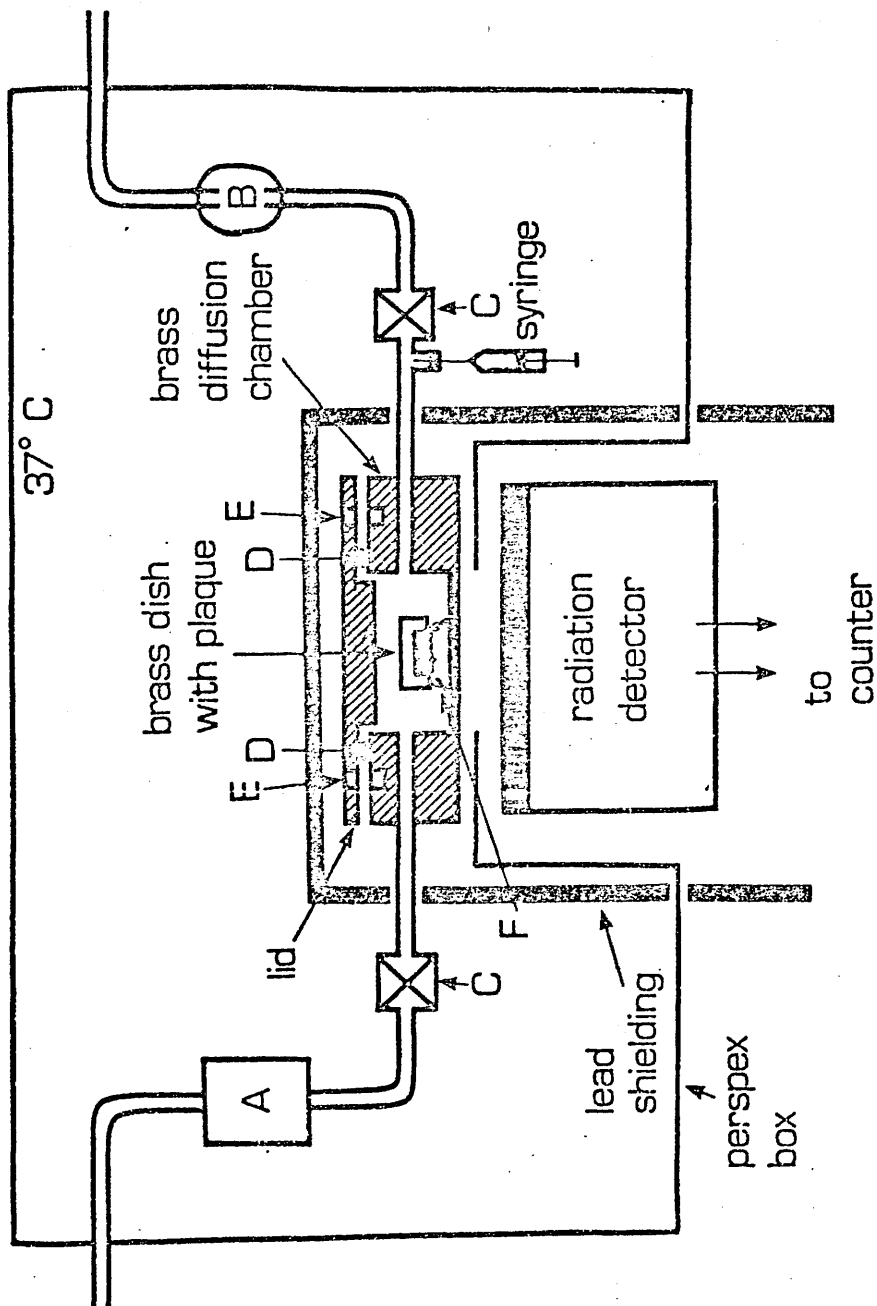


Fig. 3.2 Diagram of experimental arrangement for measuring the diffusion coefficient of xenon in plaque. A, heat exchanger and humidifier; B, air pump; C, mercury valve; D, indium wire; E, screw; F, stainless-steel wire frame.

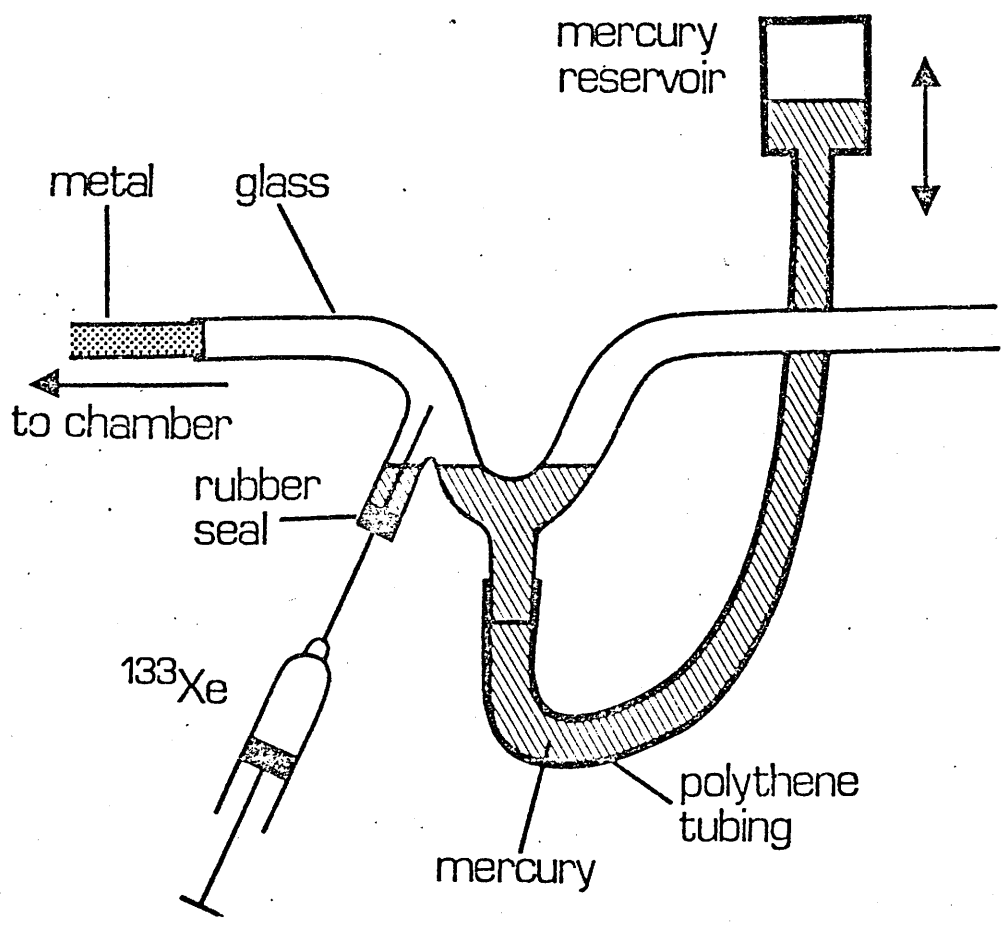


Fig. 3.3 Injection of ^{133}Xe into diffusion chamber.

plaque sample was very thin (less than 0.7 mm) all parts of it were effectively equidistant from the detector, so the counting efficiency was assumed to remain constant. Similarly, no correction was required for radioactive decay, as the radioactive half life of ^{133}Xe (5.3 days) is much greater than the time required to complete the experiment. Thus the count rate from the detector was directly proportional to the total amount of ^{133}Xe in the plaque sample.

Counts were recorded every 8 s during clearance until the count rate fell below 10 s^{-1} . This never took longer than 13 min. The chamber was then dismantled and the plaque sample thickness measured using a micrometer screw gauge (GKN Shardlow Metrology Ltd., Sheffield, England) modified by mounting a sharp tip to the end of the spindle (Fig. 3.4). An avometer (Model EM 272, AVO Ltd., Dover, England) capable of measuring electrical resistance over the range 0-10 Mohm, was used to monitor the resistance between the brass dish and the micrometer spindle. A sudden drop in resistance from infinity to about 1 Mohm precisely indicated the position of contact between the tip and the plaque surface. A further sudden decrease in resistance to 0 ohm was observed when the tip passed through the plaque and made contact with the base of the brass dish. The plaque thickness was taken to be the mean of six measurements, made at different sites on the sample.

The structure of plaque taken freshly from the mouth and from the dish at the end of the experiment was examined histologically to determine whether changes had occurred during the

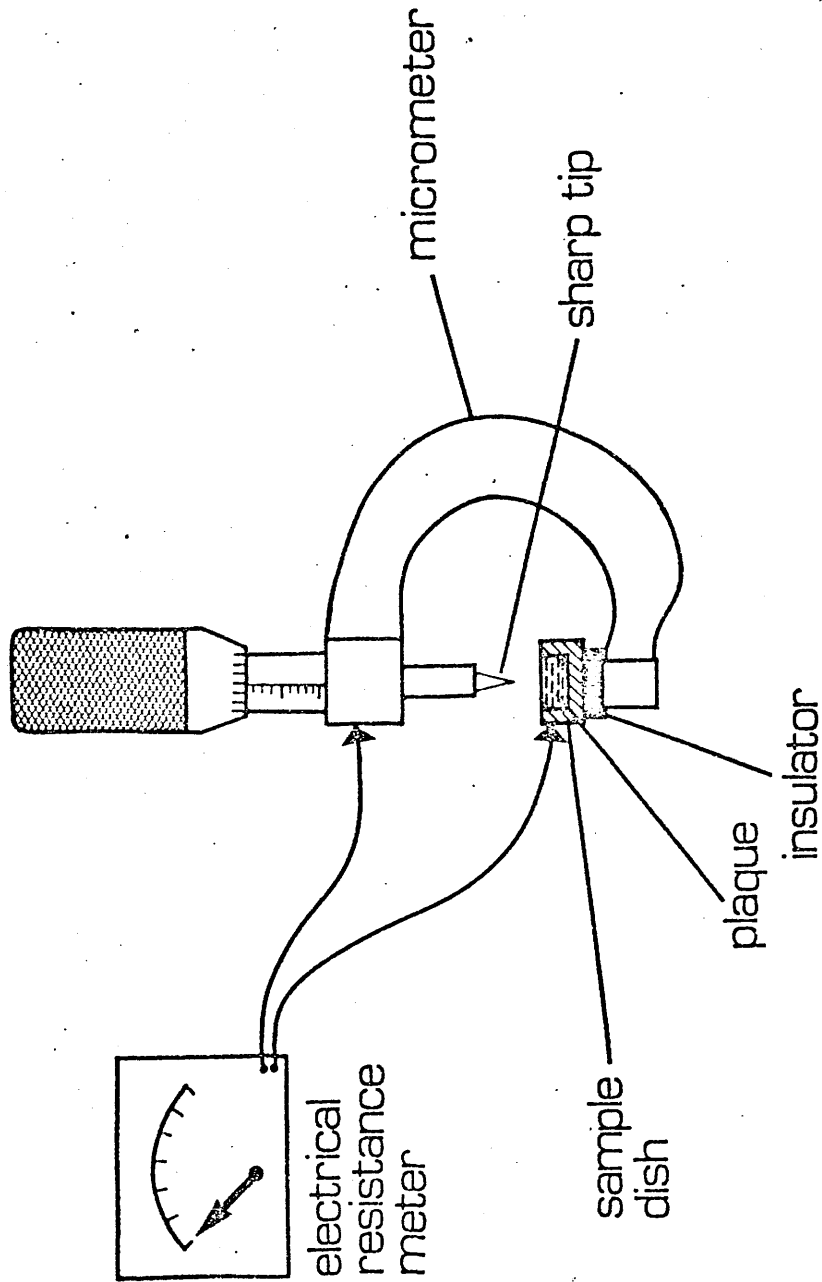


Fig. 3.4 Apparatus for measuring thickness of plaque sample.

experimental procedure. The plaque was fixed for 1.5 h in 10 ml of a 2.5 per cent solution of glutaraldehyde in 0.2 M sodium cacodylate - HCl buffer, pH 7.2, and then processed and embedded in paraffin wax and 5 μm sections cut on a rotary microtome. The plaque sections were stained with haematoxylin and eosin or a Gram stain (Brown and Brenn, 1931).

In studies of this kind, serious errors can arise from radioactive gas being adsorbed onto surfaces within the sample chamber, resulting in erroneous background counts. The system was therefore tested by monitoring the clearance of ^{133}Xe from the chamber and brass dish with no sample present. Approximately 5 mCi of ^{133}Xe were injected into the sealed chamber as before, and left for 20 min, following which the mercury valves were opened, the pump switched on and the count rate recorded.

3.4 .RESULTS

A background clearance curve (with no plaque present) is shown in Fig. 3.5, together with the clearance from a 530 μm thick disc of plaque from subject HM. With no plaque in the chamber, the count rate fell from 110,000 s^{-1} (the saturation level of the counting equipment) to 8 s^{-1} within 2 s of switching on the extraction pump. After 2 min, the count rate was 3 s^{-1} , falling exponentially to about 1 s^{-1} after 15 min. This was close to the natural background count rate of 0.5 s^{-1} .

Four such curves were averaged to give a mean background

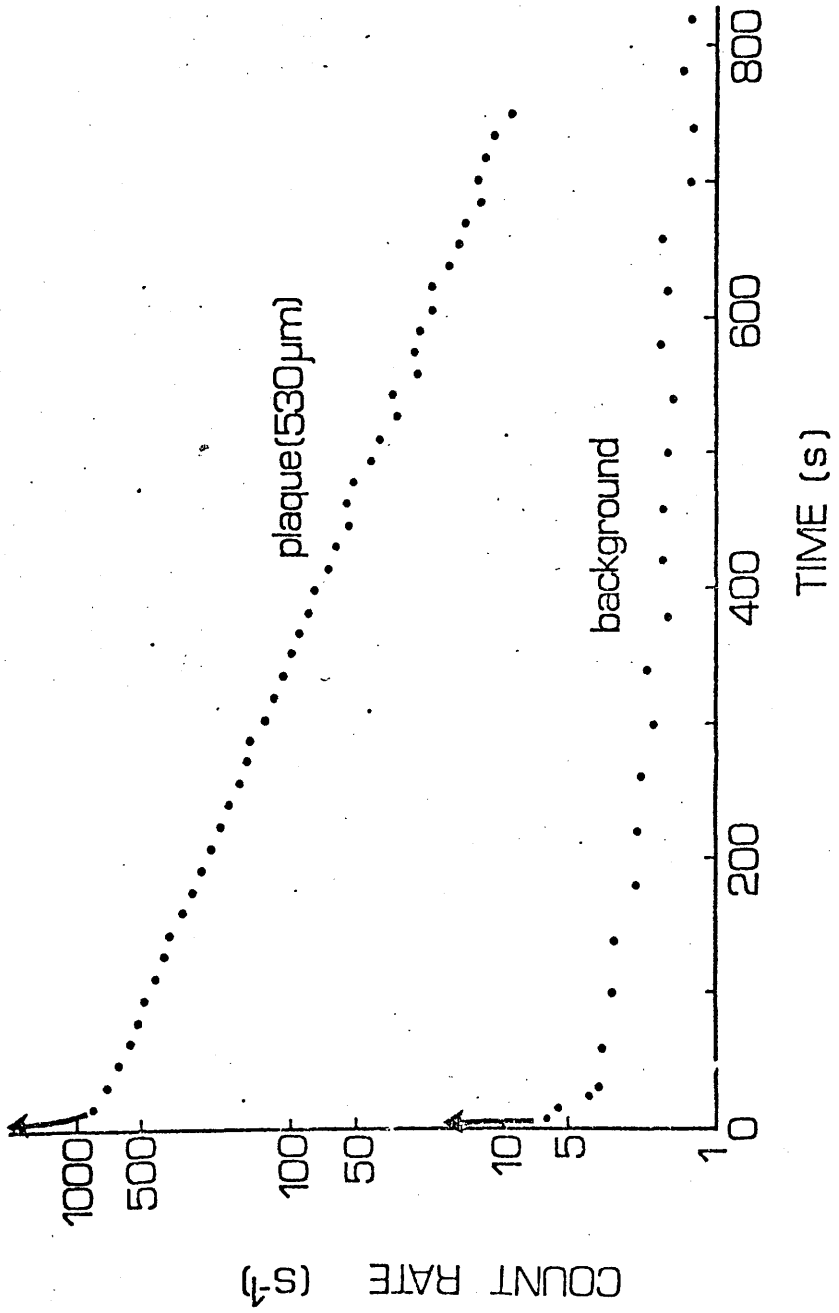


Fig. 3.5 Clearance of ¹³³Xe from the diffusion chamber in the absence of plaque (background) and from a 530 μm thick disc of plaque from subject HM.

correction curve to be subtracted from the counts obtained with plaque in the chamber. The variation between background curves was small, with a standard error of less than 10 per cent at all points on the exponential part of the curve. With plaque, an initial count rate was typically a few hundred counts per second. The background correction counts were, at a maximum, 10 per cent of the plaque counts and therefore were not insignificant, but did not introduce serious errors into the analysis because of the low standard error.

Using the method of least squares (Snedecor and Cochran, 1967) an exponential was fitted to the background - corrected clearance curve, omitting the initial phase during which there is a more rapid clearance as predicted theoretically in the higher order exponentials of Equation 3.1. From the fitted exponential, $T_{\frac{1}{2}}$ was calculated and substituted into Equation 3.4 together with the plaque thickness to yield a value for the diffusion coefficient, D , of xenon in plaque.

Table 3.1 contains details of 11 measurements of ^{133}Xe clearance from plaque with the calculated values of D . The coefficient of correlation from the least squares analysis was always greater than 0.97 ($p < 0.001$) so the uncertainty in the value for $T_{\frac{1}{2}}$ was very small. The estimated uncertainties in the values of D listed in Table 3.1 were based on the standard errors of the plaque thickness measurements and were of the order of eight per cent. Using the 11 sets of data, the mean value, \pm standard error, of D for xenon in plaque at 37°C was $(7.2 \pm 0.2) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$.

Table 3.1 The thickness of the plaque samples, the halving-times for the clearance of xenon, the correlation coefficients and the calculated values of the diffusion coefficient of xenon in plaque at 37°C.

Subject	Plaque age (days)	Plaque thickness* (μm)	$T_{\frac{1}{2}}$ (s)	Coefficient of correlation	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
SM	1	310 + 20	32.7	0.975	8.1 + 0.7
	1	350 + 20	41.6	0.989	8.3 + 0.7
	1	480 + 30	93.2	0.994	6.9 + 0.6
	1	570 + 25	157	0.974	5.8 + 0.4
	1	590 + 30	131	0.976	7.5 + 0.4
RD	1	410 + 15	60.6	0.996	7.8 + 0.4
	1	580 + 30	137	0.998	7.0 + 0.5
MM	3	580 + 20	136	0.991	6.9 + 0.3
	4	650 + 30	170	0.995	7.0 + 0.4
HM	4	530 + 12	117	0.998	6.8 + 0.2
	4	600 + 30	142	0.997	7.1 + 0.5

*Mean of six measurements + standard error.

3.5 DISCUSSION

The clearance curves show excellent agreement with the theory of diffusion from a thin layer of material. This is displayed in the high values of correlation coefficients from the regression analyses. It is difficult to show conclusively that the plaque structure had not been disturbed during the course of the experiment. No apparent drying of the plaque was observed and the histological appearance (Fig. 3.6) had not altered, indicating that the humidity was well controlled. The general appearance of Fig. 3.6 is of large areas of very densely packed bacteria and matrix, separated by less densely populated zones which occupy a small proportion of the total sample volume and would not be expected to play a significant part in the diffusion experiment. This is substantiated by the observation that, as the xenon concentration in the sample fell by two orders of magnitude, the clearance curve did not deviate from a single exponential. Thus the plaque was homogenous to the diffusion of xenon. A sample of two components, each with different diffusion characteristics, would be expected to give a multi-exponential clearance curve.

It should be noted that the 20 min allowed for equilibration is equivalent to seven or more halving-times as measured experimentally and reported in Table 3.1. As the mathematical theory applies both to equilibration and clearance, it can be calculated that sufficient time was allowed for the equilibration to reach greater than 99 per cent completion. It is unlikely that the xenon did not equilibrate throughout the entire sample. Furthermore, it should be noted from Table 3.1 that the

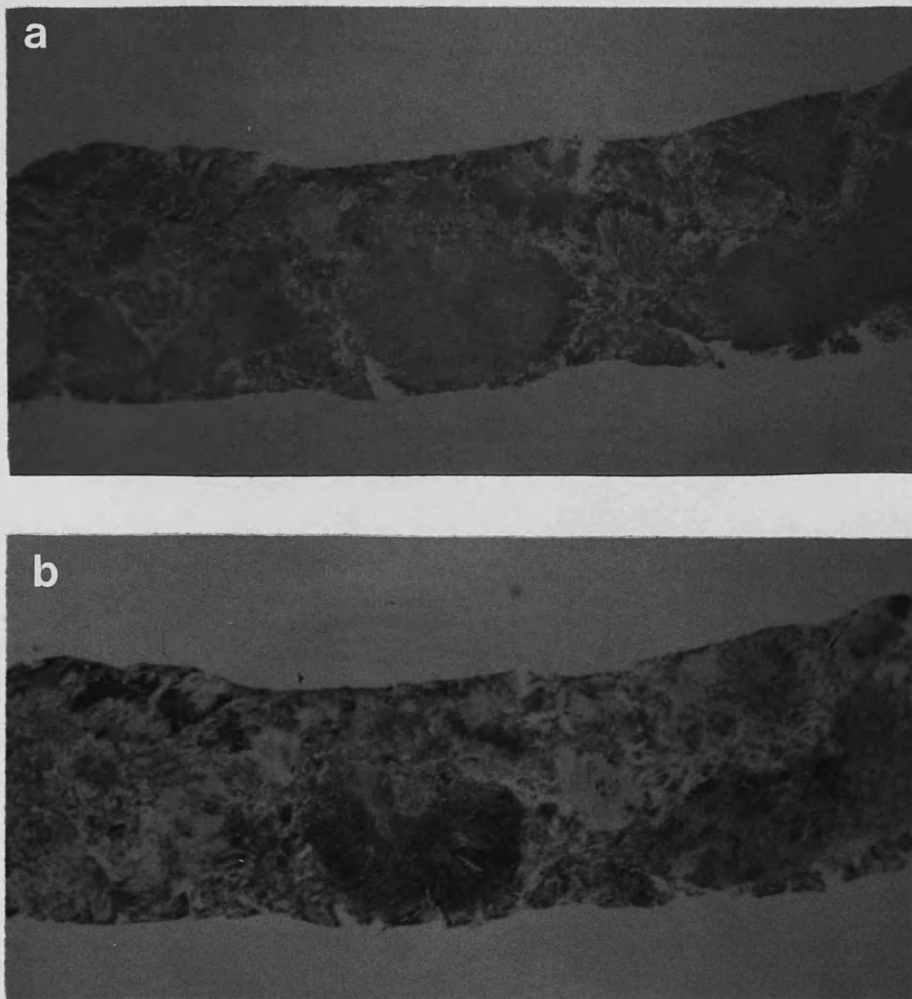


Fig. 3.6 Histological section ($5\ \mu\text{m}$) of a plaque sample after completion of xenon diffusion experiment.

Magnification $\times 66$. Staining of 3.6 a with haematoxylin and eosin and 3.6 b with Brown and Brenn.

value for D does not vary with the thickness of the plaque sample, proving that the process is not simply a surface adsorption and desorption. Thus the results should reflect the diffusion characteristics of plaque in vivo.

To establish to what extent plaque acts as a diffusion barrier, diffusion coefficients in plaque may be compared with diffusion coefficients in water. The diffusion coefficient of xenon in water at 37°C is $1.55 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Evans et al., 1974), which is twice the value measured for plaque. Using these values, the clearance of xenon from layers of plaque or water, each 100 μm thick, may be estimated. Fig. 3.7 shows that, with a $T_{\frac{1}{2}}$ of 1.8 s for water and 3.9 s for plaque, 90 per cent of the xenon will clear from the water in 6 s and from the plaque in 13 s. If the thickness of the layer is doubled, the corresponding times will increase fourfold. Diffusion of xenon in plaque is slower than in water, but a factor of two does not seem consistent with the concept that plaque forms an effective barrier to diffusion. The thickness of dental plaque found on smooth tooth surfaces in vivo is not well documented, but is most probably in the range 5 - 200 μm (Meckel, 1965; Saxton and Critchley, 1970). For such thicknesses the time-scale for diffusion of xenon is of the order of seconds.

The difference between the rates of diffusion in plaque and water can be explained by two factors. Firstly, plaque contains a high proportion of insoluble solids (70 per cent of the plaque dry weight (Jenkins, 1978, p. 371)) which will cause an increased path length for diffusion. Secondly, the extracellular

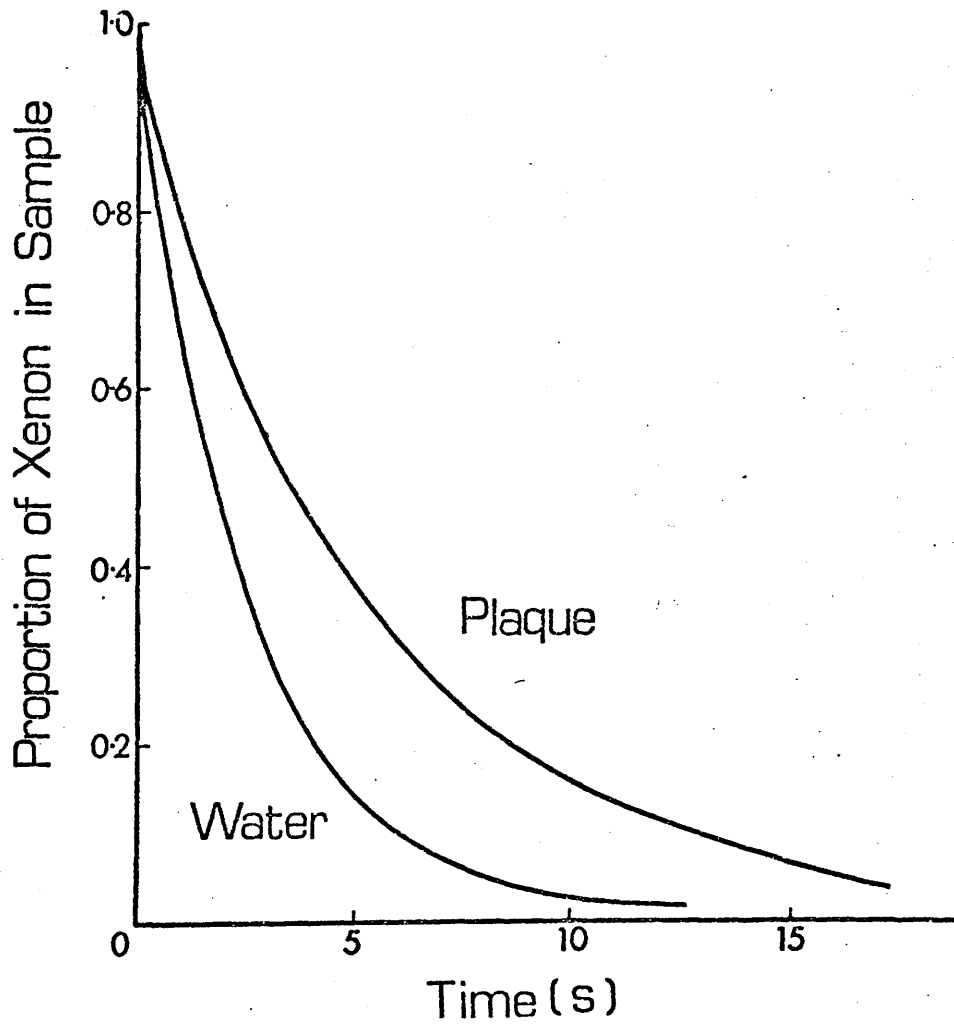


Fig. 3.7 Calculated rate of clearance of xenon from a 100 μm thick layer of plaque or water. $T_{\frac{1}{2}}$ water = 1.8 s. $T_{\frac{1}{2}}$ plaque = 3.9 s.

plaque fluid contains considerable amounts of dissolved proteins and carbohydrates (Tatevossian and Gould, 1976) making it more viscous than water. Several empirical relationships have been formulated (Reid and Sherwood, 1966) which indicate that the diffusion coefficient is inversely proportional to the viscosity of the medium.

Plaque was sampled from only four subjects for this experiment, but differences were observed in the physical characteristics of the plaque from each subject. Plaque from subject HM appeared to be stickier and of a thicker consistency than plaque from the other subjects. Likewise, plaque from subject RD appeared to be a little stickier and thicker in consistency than plaque from subjects SM and MM. However, no inter-subject variation in the value of the diffusion coefficient was detected (Table 3.1) despite a measurement precision of better than 10 per cent.

It is clear that dental plaque will not present a physical barrier to the diffusion of inert species similar in size to xenon. The penetration of substances other than xenon into plaque may be affected by metabolic, chemical or electrostatic interactions. From the measured value of the diffusion coefficient of xenon in plaque, it is possible to estimate diffusion coefficients for other gases, using Graham's law of diffusion. Graham's law states that the diffusion coefficient of a gas is inversely proportional to the square root of its molecular weight, and has been shown to apply to gases in solution as well as in the gaseous state (Jones, 1950). Table 3.2 lists estimated values of the

Table 3.2 Estimated diffusion coefficients for some gases in plaque at 37°C, using Graham's law and the experimental value for ^{133}Xe .

Gas	Molecular weight	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$	$T_{\frac{1}{2}}$ (s)	
			100 μm	200 μm
^{133}Xe	133	7.2	3.90	15.6
CO_2	44	12.5	2.25	8.99
O_2	32	14.7	1.91	7.64
N_2	28	15.7	1.79	7.16
NH_3	17	20.1	1.40	5.59

diffusion coefficients of some physiologically important gases in plaque, together with the halving times for plaque layers of 100 and 200 μm in thickness. These gases would, in the absence of interaction, diffuse into or out of such plaque layers in a few seconds. Reconciliation of the estimated rapid diffusion of oxygen with the existence of anaerobic organisms in plaque, can only be explained by rapid utilisation of oxygen by organisms near the outer surface of the plaque.

Strålfors (1950; 1956) studied the respiratory activities of oral bacteria and derived the following equation for the depth, H (cm), at which the oxygen concentration in plaque would be zero:-

$$H = \sqrt{\frac{2c D}{Q}} \quad \text{Equation 3.5}$$

where c is the concentration of oxygen in the outermost layer of plaque (arbitrary units of oxygen cm^{-3}), D is the diffusion coefficient of oxygen in plaque ($\text{cm}^2 \text{s}^{-1}$) and Q is the rate of oxygen consumption per unit volume of plaque (units of oxygen $\text{cm}^{-3} \text{s}^{-1}$). Strålfors measured Q to be $9.57 \times 10^{-5} \text{ ml O}_2 \text{ cm}^{-3} \text{ s}^{-1}$ and used the value reported by Krogh (1919) for the diffusion coefficient of oxygen in frog muscle i.e. $D = 1.4 \times 10^{-5} \text{ ml O}_2 \text{ cm}^{-2} \text{ min}^{-1}$ for a concentration gradient of 1 atmosphere cm^{-1} . For this definition of D, c is the concentration of oxygen in air (0.21 atmospheres) and so, using Equation 3.5, Strålfors calculated H to be 0.32 mm.

The diffusion coefficient for oxygen in plaque reported in Table 3.2 can be substituted into Equation 3.5 to calculate a value for H. As plaque in situ is normally covered with saliva, it will

be assumed that c is equal to the concentration of oxygen in saliva, which was reported (Long, 1961) to be $10 \text{ ml O}_2 \text{ l}^{-1}$. One mole of an ideal gas occupies a volume of 22.4 l at 1 atmosphere pressure at 0°C , and so, under these conditions, 1 ml of O_2 contains 4.46×10^{-5} moles of O_2 or 1.43 mg O_2 . Using this conversion factor, $c = 14.3 \mu\text{g O}_2 \text{ cm}^{-3}$ and the value of Q reported by Strålfors is equal to $0.14 \mu\text{g O}_2 \text{ cm}^{-3} \text{ s}^{-1}$. Substitution of these values into Equation 3.5 gives $h = 0.55 \text{ mm}$, which is slightly larger than the value reported by Strålfors.

Several assumptions have been made in the course of the above calculation, which consequently gives only an estimate of the oxygen depletion depth in plaque. The assumption that the concentration of oxygen at the outermost layer of plaque is equal to the concentration of oxygen in saliva, is not accurate because plaque contains large amounts of insoluble material. Thus, the partition coefficient i.e. the ratio of concentrations in plaque and saliva at equilibrium, will be less than unity. This suggests that the value calculated for H is an overestimate. Also, the rate of oxygen consumption by plaque bacteria is likely to show large variations according to the type and state of growth of the bacteria, the availability of nutrients, pH and possibly other factors. The respiration rates measured by Strålfors for plaque bacteria suspended in culture fluid may not be applicable to the densely packed, mixed microbial environment in plaque. A lower concentration of oxygen in saliva or a greater rate of oxygen consumption in plaque would lead to a smaller estimate for the thickness of the oxygen-containing layer in plaque. These

parameters are likely to vary according to the site in the mouth e.g. there may be differences between fissure and smooth-surface plaque, due to differences in their microbial composition and structure (Theilade, Fejerskov and Hørsted, 1976).

It is clear from the above analysis, that the proportion of anaerobic bacteria in plaque is, in part, dependent upon the rate of diffusion of oxygen in plaque. The proportion of anaerobic bacteria in plaque has been shown to increase as the plaque ages and presumably as the thickness of the plaque layer increases (Ritz, 1967; 1969). However, further investigations are necessary to establish the oxygen depletion depth in plaque in vivo.

CHAPTER 4

DIFFUSION OF SODIUM FLUORIDE IN DENTAL PLAQUE

4.1 INTRODUCTION

From the study of diffusion of xenon, it was concluded that small uncharged species can diffuse rapidly in plaque. Caries initiation and prevention will be related to diffusion in plaque of reactive substances such as sugars, acids and fluorides. The study of the rates of diffusion of these substances requires special precautions to overcome the problems of metabolic and chemical interactions. This chapter describes the measurement of the diffusion coefficient of sodium fluoride in plaque. This information helps to predict to what extent plaque will restrict the movement of fluoride into dental enamel and therefore answer the question of whether a thorough prophylaxis is essential before topical fluoride application.

4.2 MATERIALS AND METHODS

4.2.1 Plaque Source

Plaque was accumulated by three young adult subjects (SM, LS and WB) who refrained from toothcleaning for periods of 24 h. The plaque was collected as described in Section 3.3.1 and tested individually. The plaque from these subjects varied in physical appearance; plaque from subject LS was sticky and thick in

consistency and plaque from SM was relatively moist.

4.2.2 Experimental Technique

The plaque was layered into a well which had been counter-sunk into a small brass dish, similar to those described in Section 3.3.3. The plaque sample (about 12 mg) was disc-shaped with diameter 5 mm and thickness 0.57 mm. A stainless-steel wire frame was used to clamp a filter (type THWP 01300; Millipore U.K. Ltd., Abbey Road, London, England) over the plaque (Fig. 4.1). This filter, of thickness 25 μm and pore diameter 0.45 μm , held the plaque intact in the dish. The total time taken for plaque collection and insertion into this assembly was never more than 10 min. The assembly was suspended in a vessel (a Nylon liquid scintillation vial) containing 10 ml of solution with added NaF (Fig. 4.2). Twelve minutes were allowed for the NaF to diffuse into the plaque and reach equilibrium. Preliminary experiments had shown that the fluoride concentration in the plaque after this time was in excess of 80 per cent of the concentration in the equilibration solution. After 12 min the assembly was withdrawn, excess surface fluid removed from the assembly with a paper tissue, and the assembly immersed in a second vessel containing 10 ml of a NaF-free solution to allow clearance of sodium and fluoride. Both vessels were situated in an incubator maintained at 37°C and the solutions were continuously stirred using magnetic stirrers.

The subsequent clearance of sodium and fluoride from the sample was monitored by sampling 0.1 ml aliquots from the vessel at 1 min intervals for 7 min. After 7 min the assembly was

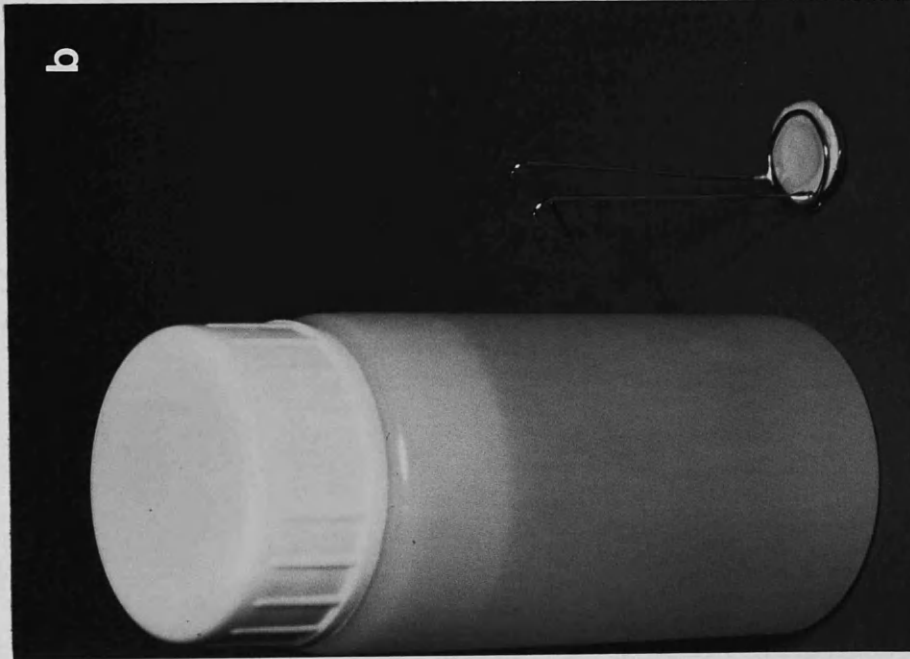
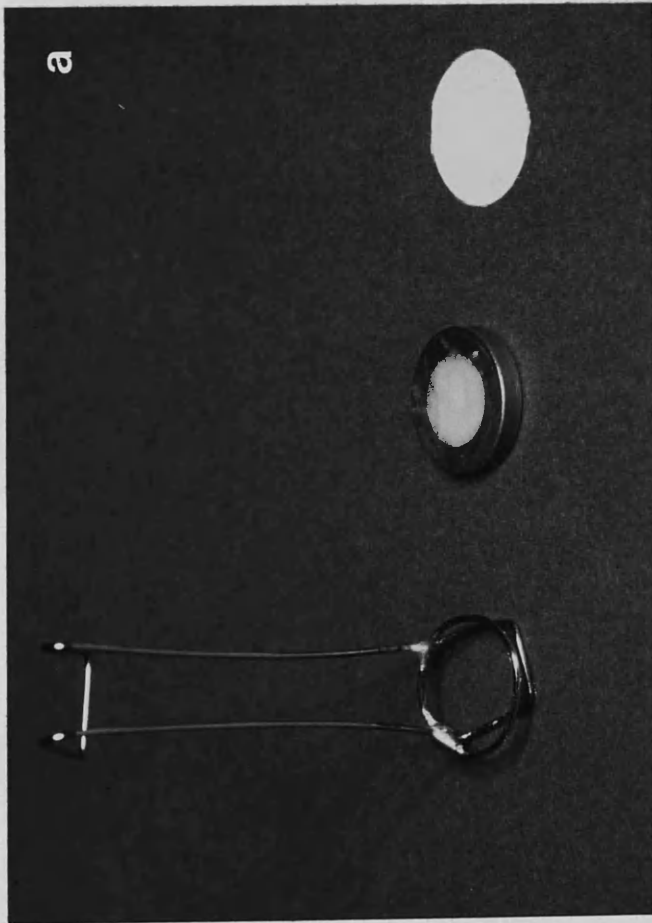


Fig. 4.1 a. Stainless-steel wire frame, brass dish containing plaque sample and a Millipore filter, for use in diffusion experiment. Magnification x 2.5.
b. Plaque assembly and equilibration vessel. Magnification x 1.5.

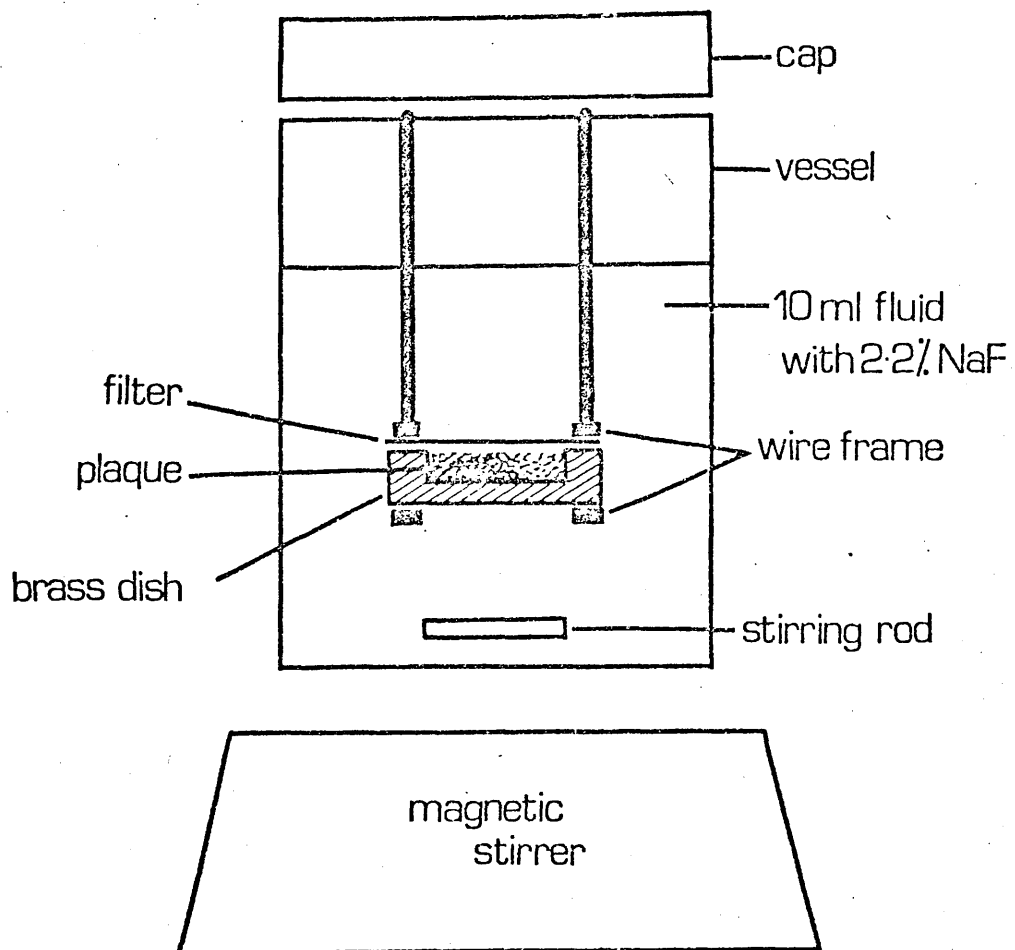


Fig. 4.2 Experimental arrangement for equilibration phase of NaF diffusion experiment.

dismantled and the filter and plaque transferred to separate polythene containers. The amount of plaque transferred and the amount left in the dish were determined by weighing on a balance (Mettler, Zürich; type H 16) to 0.01 mg. From measurements of the amount of sodium and fluoride in the plaque (corrected for total plaque weight) a clearance curve was constructed i.e. a plot of the amount of sodium or fluoride remaining within the plaque and filter against clearance time. The fluoride was assayed after dilution with 1 ml of double-distilled deionised H₂O and addition of 1 ml of TISAB II buffer (Orion, 1977) using an Orion fluoride-specific electrode with separate single-junction reference electrode (Orion, models 940900 and 900100) coupled to a digital millivolt meter (Orion, model 701A). Orion products were purchased from MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England. The sodium was determined using a flame atomic-absorption spectrometer (Instrumentation Laboratory U.K. Ltd., Birchwood, Warrington, Cheshire, England; model 151) operated in the flame-emission mode. The 0.1 ml aliquots required no pre-treatment other than dilution to 10 ml with double-distilled deionised H₂O. The plaque and filter were dried overnight in an incubator at 37°C and the Na was extracted into 0.3 ml of 7 mM K₂HPO₄ - KH₂PO₄ buffer, pH 6.8, by vortex mixing for 30 s. These samples were then centrifuged at 2000 g for 5 min at 4°C and the supernatants assayed for Na as described above.

All of the chemicals were purchased from B.D.H. Chemicals Ltd., Poole, England. The NaF was AnalaR grade and of the TISAB constituents the glacial acetic acid was AnalaR grade and the NaCl, NaOH and CDTA were laboratory grade.

Clearance of NaF from the plaque sample should be described by the same mathematical theory as was used for the xenon experiments (Section 3.2). Using the method of least squares, an exponential was fitted to the clearance curve to yield a value for the clearance halving-time, $T_{\frac{1}{2}}$. The sample thickness was not determined for each experiment as this may have led to excessive drying of the sample prior to weighing. In a preliminary experiment the thickness of plaque in the dish was measured using the micrometer screw gauge described in Section 3.3.3, for three different specimens of plaque. The mean thickness, \pm standard deviation, was 0.568 ± 0.023 mm. The sample thickness was taken to be 0.568 mm plus the thickness of the filter (0.025 mm) i.e. 0.593 mm. Using this thickness, and the value of $T_{\frac{1}{2}}$, the diffusion coefficient, D, was calculated using Equation 3.4.

As a control experiment, the transfer of fluoride from the equilibration vessel (with 2.2 per cent NaF in buffer solution) to the clearance vessel was investigated with the filter clamped to the base of the brass dish and no plaque present. The structure of the plaque at the end of the experiments was also examined histologically using the technique described in Section 3.3.3.

4.3 EXPERIMENTAL INVESTIGATIONS

4.3.1 Measurement of D for Fluoride in Plaque

Prior to each experiment, about 25 ml of unstimulated whole saliva was collected from the subject under study. The saliva

was centrifuged at 25,000 g for 20 min at 4°C and the supernatant used as the equilibration and clearance solutions, with and without the addition of 2.2 per cent NaF respectively. The clearance of fluoride from the samples was monitored as described in Section 4.2.2. The pH of the solution was not determined.

4.3.2 Variation of D for Fluoride over the Physiological pH Range of Plaque

The aim of this set of experiments was to investigate the variation of the diffusion coefficient of fluoride in plaque with pH. Instead of saliva supernatant, the following buffer solutions were used with or without 2.2 per cent NaF: 10 mM sodium acetate-acetic acid, pH 4.0 and 5.0; 7 mM K_2HPO_4 - KH_2PO_4 , pH 6.0, 6.8 and 8.0. Addition of NaF to these weak buffers resulted in a pH change. Consequently, the pH values of the equilibration solutions were 5.3, 5.8, 6.1, 6.6 and 7.4, and for the respective clearance solutions were 4.0, 5.0, 6.0, 6.8 and 8.0. The chemicals used in the preparation of these buffer solutions were AnalaR grade (B.D.H. Chemicals Ltd.).

4.3.3 Measurement of D for Sodium in Plaque

To investigate the extent to which the polarity of the electrostatic charge on the ion may affect diffusion in plaque, the clearance of sodium from plaque was monitored. This study was conducted using the 7 mM potassium phosphate buffer described above, with 2.2 per cent NaF (pH of equilibration solution = 6.6, pH of clearance solution = 6.8).

4.3.4 Dependence of D upon NaF Concentration.

Any interference from chemical interactions would be expected to become apparent as the NaF concentration was changed. For this reason, clearance of fluoride was monitored at a NaF concentration reduced 10-fold to 0.22 per cent, again using the 7 mM potassium phosphate buffer (pH of equilibration and clearance solutions = 6.8).

4.4 RESULTS

The control experiment showed that although about 80 μg of fluoride were transferred to the clearance vessel with the assembly and filter, this fluoride had reached equilibrium with the clearance solution within one minute. This demonstrates that the rate of stirring of the solution was satisfactory, and that the fluoride rapidly diffused out of the filter.

A typical clearance curve (from subject WB in study 4.3.1) is shown in Fig. 4.3. The amount of fluoride (in μg) remaining in the plaque, is plotted on a logarithmic scale against clearance time. Linear regression analysis indicates a highly significant correlation ($r = 0.99$, $p < 0.001$) on this scale, which means that the clearance curve is well described by a single exponential, as expected theoretically. For the other measurements it was found that $r \geq 0.96$ and $p < 0.001$. The values of $T_{\frac{1}{2}}$ were calculated from the regression analyses.

The experimental results are summarised in Tables 4.1 to 4.4. For the study described in Section 4.3.1, the mean value

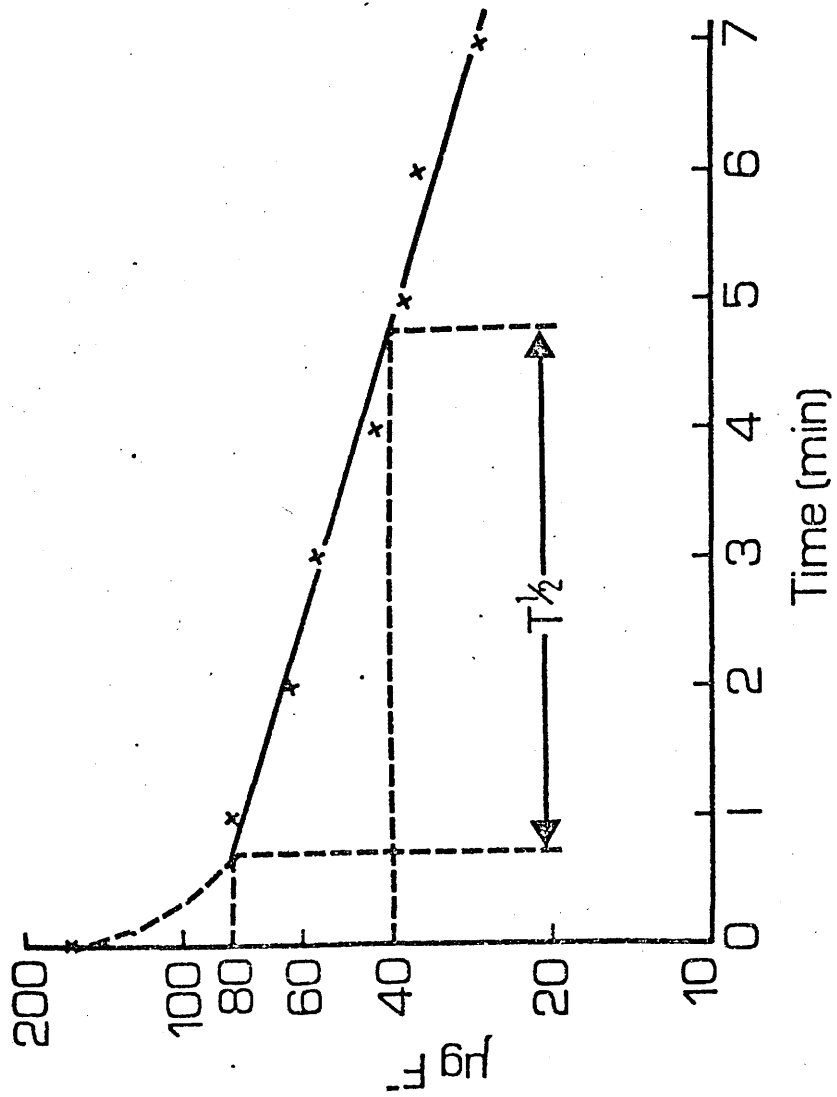


Fig. 4.3 Clearance of fluoride from plaque sample from subject WB, under conditions described in Section 4.3.1.

of D for fluoride in plaque, \pm standard error (S.E.), was found to be $(4.09 \pm 0.26) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Table 4.1). D did not vary significantly between the plaque from each subject. Table 4.2 indicates that there was no significant variation in D over the pH range investigated. The pH of the clearance solutions remained constant during the clearance phase.

For sodium, the mean value measured for D, \pm S.E., was $(4.09 \pm 0.14) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Table 4.3), which is identical to the value found for fluoride. When the fluoride concentration was reduced ten-fold, the mean value of D for fluoride did not change significantly (mean value = $4.12 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, Table 4.4).

Using the 22 fluoride measurements listed in Tables 4.1, 4.2 and 4.4, the mean value of the diffusion coefficient, \pm S.E., for fluoride in dental plaque at 37°C was $(4.24 \pm 0.11) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$.

4.5 DISCUSSION

By monitoring clearance from, rather than penetration into plaque, many of the problems of chemical interaction are circumvented. Several studies have shown that fluoride binds strongly to plaque bacteria. In a review article, Jenkins and Edgar (1977) report plaque fluoride concentrations in the range 1 - 179 parts per million (wet weight), which are much lower than the 1,000 and 10,000 parts per million fluoride used in the equilibration solutions in this study. The fluoride-binding sites

Table 4.1 Diffusion coefficients, D, for fluoride in plaque, measured under the conditions described in Section 4.3.1.

$T_{\frac{1}{2}}$ = clearance halving time.

Subject	$T_{\frac{1}{2}}$ (s)	D ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
SM	306	3.22
	217	4.56
	256	3.86
	186	5.30
LS	275	3.59
	278	3.56
WB	243	4.06
	216	4.57

mean D, \pm S.E. = $(4.09 \pm 0.26) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$

Table 4.2 Variation with pH of D for fluoride in plaque
(see Section 4.3.2).

Equilibration pH	Clearance pH	Subject	$T_{\frac{1}{2}}$ (s)	D ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
5.3	4.0	SM	241	4.10
		SM	227	4.36
5.8	5.0	SM	239	4.14
		WB	213	4.65
6.1	6.0	SM	235	4.20
		SM	203	4.87
6.6	6.8	SM	235	4.20
		SM	193	5.11
		SM	217	4.56
7.4	8.0	SM	230	4.29
		SM	264	3.74

$$\text{mean } D \pm \text{S.E.} = (4.38 \pm 0.12) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$$

Table 4.3 D for sodium in plaque (Section 4.3.3).

Subject	$T_{\frac{1}{2}}$ (s)	D $(\text{cm}^2 \text{s}^{-1}) \times 10^6$
WB	240	4.11
SM	258	3.83
SM	224	4.41
SM	246	4.02

mean D \pm S.E. = $(4.09 \pm 0.14) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$

.....
Table 4.4 D for fluoride in plaque using 0.22 per cent NaF (Section 4.3.4).
.....

Subject	$T_{\frac{1}{2}}$ (s)	D $(\text{cm}^2 \text{s}^{-1}) \times 10^6$
SM	232	4.26
SM	240	4.11
SM	248	3.99

mean D = $4.12 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$

within the plaque should become saturated during the equilibration phase of this study, and this bound fluoride will play no part in the clearance process. The mean $T_{\frac{1}{2}}$ value for these experiments was 237 s. Thus, 12 min were sufficient for about 90 per cent equilibration, which ensures a very high and sufficiently uniform NaF concentration within the plaque. If a very slow diffusion phase does exist in plaque it involves a maximum of 10 per cent of the NaF and is therefore not significant.

The histological sections of the plaque at the end of the experiment (Fig. 4.4) closely resemble the sections shown in Fig. 3.6, and again indicate that the plaque remained relatively intact throughout the experiment. The clearance curves did not depart from a single exponential during the 7 min clearance phase, during which the plaque fluoride concentration fell ten-fold. The plaque samples appear to be homogeneous to the diffusion of NaF.

The measured diffusion coefficient of NaF in plaque at 37°C was $4.2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, and although this is about a quarter of the diffusion coefficient of NaF in water (D for NaF in water = $1.87 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$; see Appendix), it indicates that diffusion of fluoride in plaque is rapid. Using this value of D , the $T_{\frac{1}{2}}$ for a 100 μm layer of plaque would be 6.7 s. Fig. 4.5 shows the calculated rate of penetration of NaF into a 100 μm layer of plaque, assuming no chemical interaction. Equilibration would be 80 per cent complete after 15 s. Thus the time scale for diffusion through layers of plaque of the thickness expected to accumulate on tooth smooth surfaces within 24 h, is a matter of seconds. It should

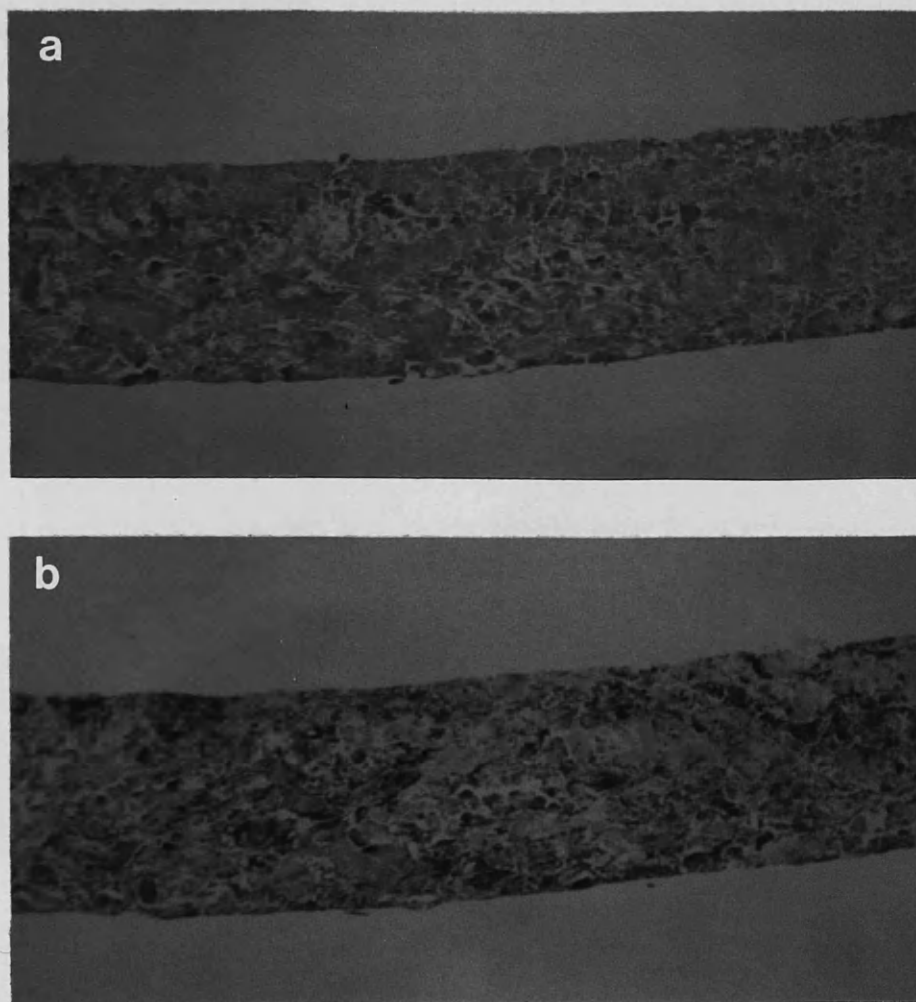


Fig. 4.4 Histological sections ($5\ \mu\text{m}$) of a plaque sample after completion of NaF diffusion experiment.

Magnification x 50. Staining of 4.4 a with haematoxylin and eosin and 4.4 b with Brown and Brenn.

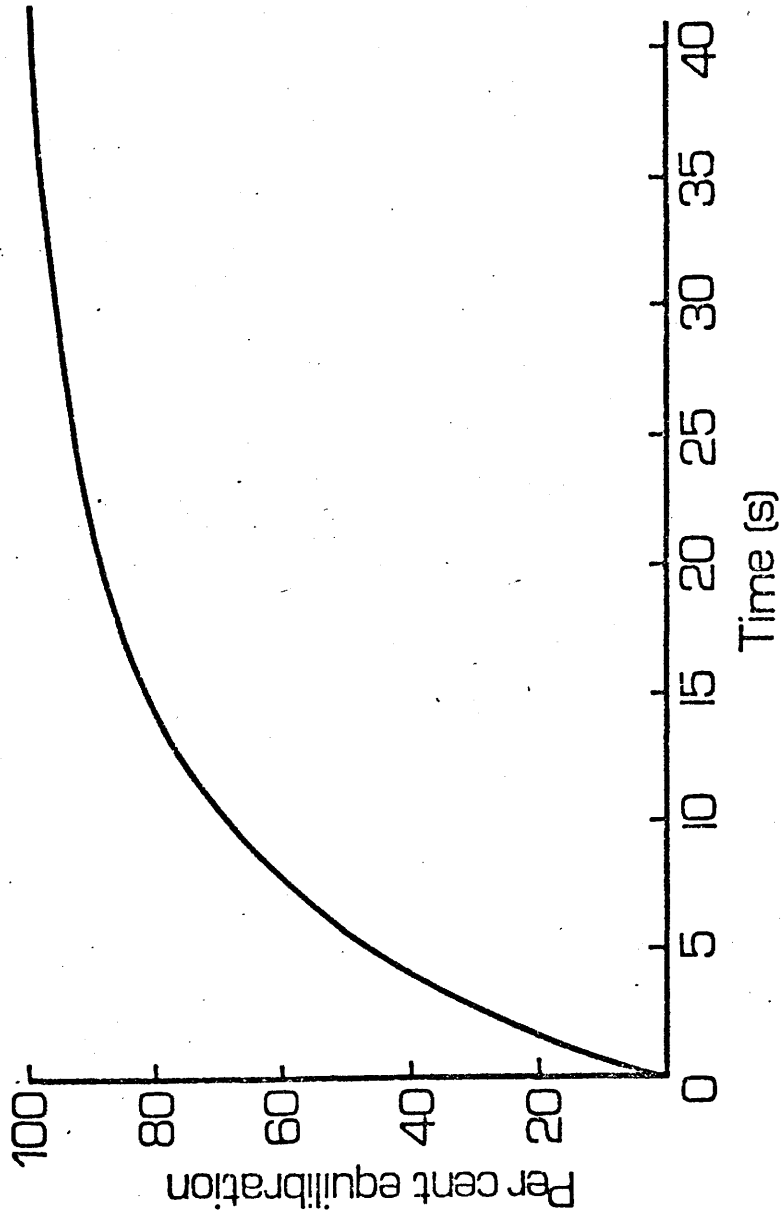


Fig. 4.5 Calculated rate of penetration of NaF into a 100 μm thick layer of plaque assuming no metabolic or chemical interaction ($T_{1/2} = 6.7$ s).

be borne in mind that the diffusion time is proportional to the square of the thickness of the plaque layer (Equation 3.4). For a layer of plaque 0.5 mm thick the diffusion time extends to about 5 min. Thus it may take a few minutes for NaF to fully penetrate plaque in interproximal sites where plaque accumulates to greater thicknesses.

The finding that the sodium and fluoride ions diffuse in plaque at the same rate reflects the need for electrostatic balance. This may seem to be at variance with the conclusion of Melsen et al. (1979) who suggested that plaque is permselective. However, they studied penetration of radio-labelled calcium, phosphate, glucose and glucosamine into columns of plaque and their measurements were subject to the effects of chemical binding and perhaps iso-ionic exchange of ^{45}Ca and ^{32}P with stable calcium and phosphate. Using a different technique to measure diffusion coefficients in plaque, Dibdin (1979) concluded that plaque exhibited only limited permselectivity, although acids diffused slightly faster below their pK_a 's. In the study described in Section 4.3.2, no variation was found in the diffusion coefficient of fluoride over a range of plaque pH (Table 4.2), but even at the lowest of the equilibration pH values (5.3), NaF is more than 98 per cent dissociated (pK_a for $\text{HF} = 3.45$; Weast, 1975).

In the complex oral environment, chemical interactions may be of greater importance in determining the rate of fluoride penetration into, and clearance from plaque, especially for low fluoride concentrations. Plaque may also affect the fluoride-

enamel reactions e.g. the relative amounts of fluorapatite and CaF_2 formation. However, the rapid rate of diffusion of fluoride through thin layers of plaque suggests that the effectiveness of topical fluoride applications, by gels, mouthrinses, tablets, etc., may not be diminished by a thin plaque-coating on the teeth. One important parameter to consider is the fluoride concentration achieved at the plaque-enamel interface relative to that achieved at the saliva-enamel interface in the absence of plaque. Tatevossian (1978) reported that the fluoride-ion activity, $a\text{F}^-$, in plaque fluid 1 min after completion of a 1 min mouthrinse with 0.02 M NaF (380 ppm fluoride) was only 30 per cent of the $a\text{F}^-$ in the saliva-mouthrinse mixture expectorated at the end of the rinse. However, considerable amounts of fluoride must have diffused back out of the plaque in the 1 min time lapse between the end of the mouthrinse and the start of plaque collection. By extrapolation of Tatevossian's data, using the mathematical theory detailed in Section 3.2 and making allowance for the fact that plaque collection must have taken at least 2 min (Tatevossian, personal communication) it can be shown that the $a\text{F}^-$ in the plaque fluid at the end of the rinse must have been at least 80 per cent of the $a\text{F}^-$ in the saliva-mouthrinse mixture.

Rapid diffusion of fluoride through thin layers of plaque, together with the observation that the $a\text{F}^-$ in the plaque fluid is not substantially less than the $a\text{F}^-$ in the saliva, suggests that a thorough prophylaxis may not be necessary prior to topical fluoride treatments and that efficient oral hygiene on the part of the patient may be sufficient. This is consistent with the findings of Kirkegaard et al. (1975) who showed that a coating of artificial

plaque did not significantly reduce the amount of fluoride taken up by enamel from a two per cent NaF solution in vitro. Bruun and Stoltze (1976) also showed that a five-day growth of plaque did not reduce the amount of fluoride taken up by enamel from NaF or amine F solutions in vivo and Joyston-Bechal, Duckworth and Braden (1976) showed that artificially-produced pellicle and plaque did not reduce the amount of ¹⁸F taken up by enamel from NaF or acidulated phosphate fluoride solutions in vitro. Tinanoff, Wei and Parkins (1974; 1975) have also demonstrated that a pellicle-coating on enamel did not significantly reduce the uptake of fluoride from acidulated phosphate fluoride solution in vivo or in vitro.

CHAPTER 5

RATES OF DIFFUSION OF SUGARS IN PLAQUE

5.1 INTRODUCTION

Stephan (1940) showed that following a short exposure to glucose, the pH of plaque fell rapidly to about 5.0 and then took a long time, often more than 30 min, to return to the initial pH level. This 'Stephan curve', which has been reproduced on numerous occasions under a variety of conditions, is difficult to analyse in dynamic terms. There are at least four important parameters to consider. Firstly, the rate of diffusion of sugars from saliva into plaque. Secondly, the rate at which plaque bacteria can metabolise the sugars to form acid. Thirdly, the rate of diffusion of acid from plaque. Fourthly, the salivary flow rate, which is important for two reasons. Saliva has the capacity to raise the pH of plaque by a buffering action and saliva washes sugars and acids from the mouth.

Clearly the rate-controlling step of the process could be the rate of penetration of sugar into plaque, if this resulted in a limitation of substrate for metabolism. The aim of the study reported in this chapter was to measure the diffusion coefficient of sucrose in plaque using a modification of the technique described in Chapter 4. In order to do this, precautions must be taken to prevent metabolism of the sucrose. This was achieved by fixation of the plaque with glutaraldehyde - a fixative used routinely in the preparation of pathology specimens for examination by electron

microscopy. Experiments were performed to show that fixation prevented lactate production from sucrose by plaque. The effect of fixation on diffusion was assessed by measuring the diffusion coefficients of NaF and of xylitol (a polyol which is not rapidly metabolised by plaque bacteria) in plaque and in glutaraldehyde-fixed plaque.

5.2 MATERIALS AND METHODS

5.2.1 Source of Plaque

Plaque for these experiments was collected from a group of five subjects (SM, LJ, DW, HMA and LS) who refrained from tooth-cleaning for periods of 24 h. Plaque was collected as described in Section 3.3.1 and tested individually.

5.2.2 Diffusion of NaF in Glutaraldehyde-Fixed Plaque

The diffusion coefficient of NaF in glutaraldehyde-fixed plaque was measured using a technique similar to that described in Section 4.2. Plaque was layered into the brass dish and a filter was clamped into position, as before. The plaque was then fixed by immersing the assembly for 1.5 h at room temperature in 5 ml of a 2.5 per cent solution of glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer, pH 7.2. These chemicals were laboratory grade (B.D.H. Ltd.).

The assembly was then equilibrated for 12 min in 10 ml of a 2.2 per cent solution of NaF in 7 mM KH_2PO_4 - K_2HPO_4 buffer, pH 6.8

(resultant pH with NaF = 6.6). The assembly was next transferred to 10 ml of NaF-free potassium phosphate buffer and clearance of fluoride from the plaque was monitored as in Section 4.2 by sampling 0.1 ml aliquots of the buffer at 1 min intervals for 7 min. The equilibration and clearance solutions were maintained at 37°C and continuously stirred. The 0.1 ml aliquots and the plaque and filter at the end of the clearance were assayed for fluoride as before, using Orion fluoride-specific electrodes, and a clearance curve was plotted.

5.2.3 Diffusion of ¹⁴C-Sucrose in Glutaraldehyde-Fixed Plaque.

Uniformly labelled ¹⁴C-sucrose was purchased as a crystalline solid in 50 µCi batches from two commercial sources. The New England Nuclear product was obtained through their European agents, NEN Chemicals GmbH, Dreieichenhain, West Germany. The specific activity was 3.60 mCi per mmole and radiochemical and chemical purity was greater than 99 per cent. The ICN product was obtained from Laboratory Impex Ltd., Middlesex, England. The specific activity was 12.95 mCi per mmole and radiochemical and chemical purity was greater than 98 per cent. Each 50 µCi batch of ¹⁴C-sucrose was dissolved in 1 ml of the 7 mM potassium phosphate buffer, pH 6.8, to provide a stock radioisotope solution.

After fixation in glutaraldehyde, the plaque assembly was equilibrated for 40 min in 10 ml of the potassium phosphate buffer containing 100 mM unlabelled sucrose and 0.4 ml of the stock ¹⁴C-sucrose solution (20 µCi). The assembly was then transferred to 10 ml of sucrose-free potassium phosphate buffer and clearance of

^{14}C -sucrose from the plaque was monitored by sampling 0.1 ml aliquots of the buffer 2, 3, 4, 5, 6, 8, 10, 12, 14, 17 and 20 min after immersion. These samples were placed in liquid scintillation vials. After 20 min clearance, the assembly was dismantled and the filter and plaque transferred to separate vials. The amounts of plaque transferred to the vial and left in the dish were determined by weighing to 0.01 mg on a precision balance (Type H16, Mettler, Zürich). During equilibration and clearance, the solutions were maintained at 37°C and continuously stirred.

Ten ml of a xylene-based liquid scintillant (NE260; Nuclear Enterprises Ltd., Edinburgh, Scotland) were added to each of the vials prior to counting on a Packard Model 3320 Tri-Carb liquid scintillation counter (Packard Instrument Company Inc., Illinois, USA). Samples were counted for at least 50 min producing in excess of 15,000 counts. The counting efficiency of each sample was determined using the 'spiking' technique (Neame and Homewood, 1974) i.e. 0.5 μCi of ^{14}C -sucrose (equivalent to 18,500 disintegrations per second) were added to each sample and the samples were recounted. The counting efficiency was calculated by dividing the observed increase in count rate by 18,500.

5.2.4 Diffusion of ^{14}C -Xylitol in Plaque and Glutaraldehyde-Fixed Plaque.

Uniformly labelled ^{14}C -xylitol was purchased in 50 μCi batches from the Radiochemical Centre, Amersham. It was supplied in aqueous solution (approximately 1 ml) with three per cent ethanol. The specific activity was 87.5 mCi per mmol and the radiochemical purity was 99 per cent. The ethanol was removed by evaporation

under a stream of nitrogen to approximately 50 per cent volume. A stock ^{14}C -xylitol solution was then prepared by adding 2 ml of the 7 mM potassium phosphate buffer, pH 6.8.

Diffusion of ^{14}C -xylitol in plaque at 37°C was studied with and without prior fixation of the plaque sample in the 2.5 per cent glutaraldehyde solution. The experimental technique was the same as that used for ^{14}C -sucrose. The equilibration solution consisted of 100 mM xylitol (B.D.H. Ltd.) in the 7 mM potassium phosphate buffer with approximately 10 μCi ^{14}C -xylitol (0.5 ml of the stock solution). Samples were counted for at least 50 min, producing in excess of 5000 counts. The specific activity of the stock solution was not accurately known, but the relative counting efficiency of the sample was established using the spiking technique.

As the theory outlined in Section 3.2 is expected to apply to these experiments, clearance curves were plotted on semi-logarithmic graph paper i.e. the logarithm of the amount of fluoride, sucrose or xylitol within the plaque was plotted against clearance time. Values of clearance halving-time, $T_{\frac{1}{2}}$, obtained by regression analysis were substituted into Equation 3.4 to yield values for the diffusion coefficients.

5.2.5 Effect of Glutaraldehyde-Fixation on Lactate Production in Dental Plaque.

A control experiment was performed to establish whether

fixation with glutaraldehyde prevented acid production from sucrose by plaque. Two subjects (HMA and LS) accumulated in excess of 25 mg of plaque in 24 h so it was possible to measure their plaques individually. For each of these subjects, plaque was collected as described in Section 3.3.1 and used to fill two of the plaque assemblies. One assembly was incubated, with continuous stirring, for 40 min at 37°C in 10 ml of 7 mM potassium phosphate buffer, pH 6.8, containing 100 mM sucrose. The second assembly was fixed in glutaraldehyde for 1.5 h before incubation in a similar sucrose solution. This incubation is identical to the equilibration phase of the ¹⁴C-sucrose experiments except that no labelled sucrose was present. At the end of the incubation, each of the sucrose-buffer mixtures were assayed for lactate by the technique of isotachopheresis using an LKB 2127 Tachophor (LKB Produkter AB, Bromma, Sweden). Assay of lactate and acetate by isotachopheresis will be described in detail in Section 6.2.2. In the present study, 20 µl of each solution were injected into the tachophor capillary without pretreatment.

5.3 RESULTS

The concentrations of lactate in the sucrose-containing buffers after incubation with plaque or glutaraldehyde-fixed plaque are listed in Table 5.1. Without fixation, the lactate concentrations were 0.14 and 0.12 µmol/ml for subjects HMA and LS, respectively. With fixation, the lactate concentration in each case was below the limit of detection of 0.01 µmol/ml.

The liquid scintillation counting efficiency was about

Table 5.1 Lactate concentrations in sucrose-containing buffer incubated with plaque and glutaraldehyde-fixed plaque.

Subject	lactate concentration ($\mu\text{mol/ml}$)	
	plaque	fixed-plaque
HMA	0.14	N.D.
LS	0.12	N.D.

N.D. not detectable i.e. $< 0.01 \mu\text{mol/ml}$

90 per cent and did not vary between samples. All of the clearance curves from the diffusion experiments were single exponential and regression analysis revealed highly significant correlations ($r \geq 0.95$, $p < 0.001$). Fig. 5.1 is a typical clearance curve for ^{14}C -sucrose.

Individual measurements of diffusion coefficients are listed in Table 5.2 and the mean values of the diffusion coefficients of NaF, ^{14}C -sucrose and ^{14}C -xylitol in plaque and glutaraldehyde-fixed plaque are listed in Table 5.3. Both NaF and ^{14}C -xylitol diffused slightly faster in the fixed plaque but the increases were not of statistical significance (for NaF, $t = 1.339$, $p > 0.1$; for xylitol, $t = 1.857$, $p > 0.1$; Student's t-test).

The mean diffusion coefficient of ^{14}C -sucrose in glutaraldehyde-fixed plaque at 37°C , \pm standard deviation, was $(3.0 \pm 0.3) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, which is about the same as the diffusion coefficient of ^{14}C -xylitol in fixed plaque.

5.4 DISCUSSION

Glutaraldehyde acts by cross-linking proteins and should therefore inactivate the enzymes involved in the metabolism of sucrose without causing extreme changes in the plaque structure. The results in Table 5.1 show that fixation with glutaraldehyde effectively prevents acid production from sucrose by plaque. Without fixation, about 117 nmoles of lactate were produced per mg of plaque. This is consistent with the results of Geddes (1972) who

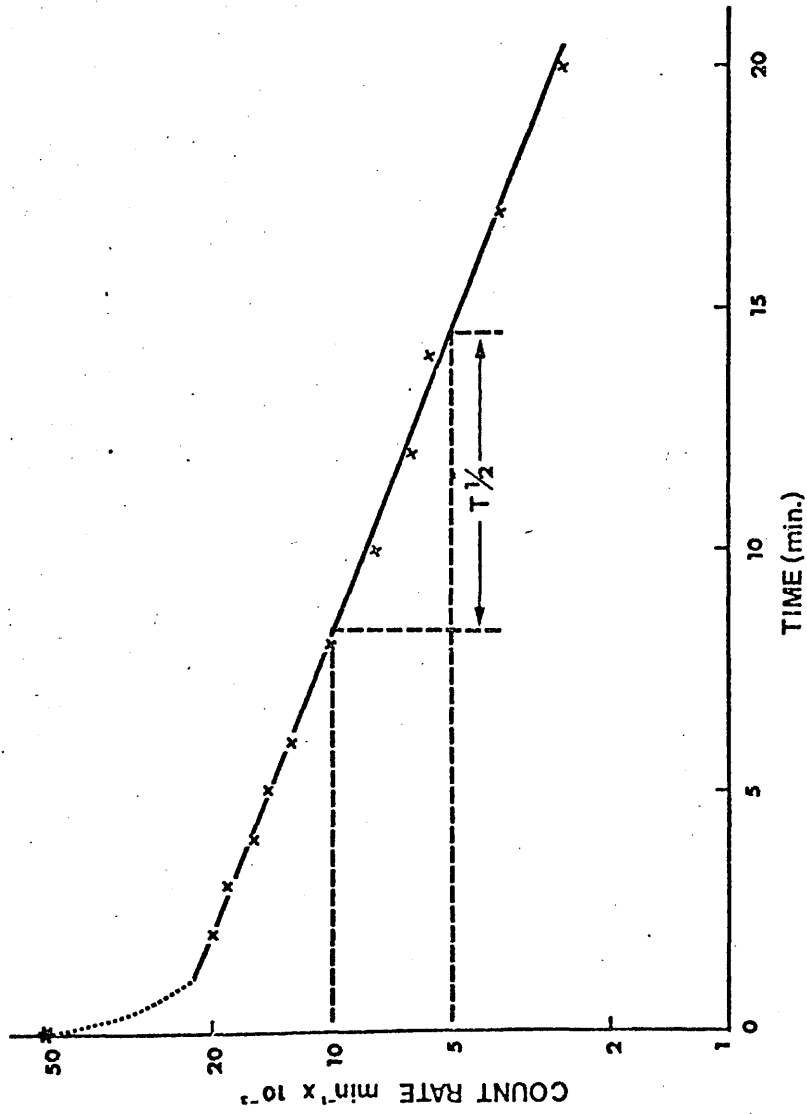


Fig. 5.1 Clearance of ^{14}C -sucrose from a glutaraldehyde-fixed plaque sample from subject SM.

Table 5.2 Measurements of diffusion coefficients of NaF, ^{14}C -sucrose and ^{14}C -xylitol in plaque and glutaraldehyde-fixed plaque at 37°C .

Diffusing substance	Subject	$T_{\frac{1}{2}}$ (s)	D ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
(a) Plaque			
^{14}C -xylitol	LJ	408	2.42
	SM	399	2.48
	DW	487	2.03
	SM	429	2.30
(b) Glutaraldehyde-fixed plaque			
NaF	SM	220	4.49
	SM	273	3.62
	SM	169	5.84
	DW	205	4.81
	SM	209	4.73
^{14}C -xylitol	SM	413	2.39
	SM	311	3.18
	SM	412	2.40
	SM	302	3.27
^{14}C -sucrose	SM	297	3.33
	SM	351	2.81
	SM	327	3.02
	SM	369	2.68

Table 5.3 Mean values of the diffusion coefficients of NaF, ^{14}C -sucrose and ^{14}C -xylitol in plaque and glutaraldehyde-fixed plaque.

Diffusing substance	D ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$	
	plaque	glutaraldehyde-fixed plaque
NaF	4.2 \pm 0.5* n = 22	4.7 \pm 0.8 n = 5
^{14}C -sucrose	-	3.0 \pm 0.3 n = 4
^{14}C -xylitol	2.3 \pm 0.2 n = 4	2.8 \pm 0.5 n = 4

Values listed as means \pm standard deviations

n = number of measurements

* from Chapter 4

found that 110 nmoles of lactate were produced per mg of plaque during 15 min incubation in 150 mM sucrose solution. However, with fixed plaque, any lactate produced was below the limit of detection i.e. less than 8.3 nmoles per mg plaque

The results in Table 5.3 show that fixation of plaque with glutaraldehyde does not significantly affect the rates of diffusion of NaF or xylitol. The sodium and fluoride ions are both small and might be expected to reflect any change in the charge distribution within the plaque. The xylitol molecule carries no net charge and more closely resembles the size and structure of sucrose. There is considerable evidence to show that xylitol is not rapidly metabolised by plaque bacteria (Muhlemann and de Boever, 1970; MacFadyen et al. 1976; Mäkinen, 1978; Beeley et al., 1979) and that it does not bind in significant amounts to plaque components (Mäkinen and Rekola, 1976). It is therefore concluded that the measured value of the diffusion coefficient of ^{14}C -sucrose in glutaraldehyde-fixed plaque, $(3.0 \pm 0.3) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, is a good estimate of the diffusion coefficient of sucrose in plaque. This is similar to the value found by Dibdin (1979, and personal communication) using a different technique, for the diffusion coefficient of sucrose in heat-killed plaque (see Chapter 10 for further details).

The diffusion coefficient of sucrose in water at 37°C is about $7.0 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (see Appendix). The ratio of the diffusion coefficient of sucrose in plaque to the diffusion coefficient of sucrose in water is therefore 2.3, which is intermediate between the value of 2.15 found for the inert xenon, and 4.4 found for the

strong electrolyte NaF. However, it should be noted that sucrose diffuses more slowly than NaF in plaque.

Using these values, the half-lives, $T_{\frac{1}{2}}$, for the diffusion of NaF, sucrose and xylitol into plaque layers 50, 100 and 200 μm in thickness have been calculated (Table 5.4). For 50 μm , the values of $T_{\frac{1}{2}}$ are between 1 and 3 s. The diffusion process is extremely rapid and would reach equilibrium within about 10 s. When the plaque thickness is doubled the $T_{\frac{1}{2}}$ values increase four-fold. Fig. 5.2 illustrates the calculated rate of penetration of NaF, sucrose and xylitol into a 100 μm plaque layer assuming no metabolic or chemical reactions. Sucrose equilibration would reach 80 per cent completion within 20 s. Increasing the thickness to 200 μm gives a corresponding increase in diffusion time, but 70 per cent equilibration would still be achieved within 1 min.

For steady state conditions, the maximum rate of flow of sucrose into a layer of plaque will occur when the rate of metabolism of sucrose by plaque bacteria is equal to the flow rate, so that the sucrose concentration at the base of the plaque layer is zero. (If the rate of metabolism was less than the rate of penetration, the sucrose concentration in the plaque would increase, thus reducing the concentration gradient and therefore the flow rate.) This situation is equivalent to the simultaneous diffusion and metabolism of oxygen in plaque, discussed in Section 3.5 and described mathematically by Equation 3.5:-

$$H = \sqrt{\frac{2c D}{Q}}$$

In this case, H is the thickness of the plaque layer (cm),

Table 5.4 Calculated values of $T_{1/2}$ for NaF, sucrose and xylitol for 50, 100 and 200 μm thick layers of plaque, assuming no metabolic or chemical interactions.

Diffusing substance	$T_{1/2}$ (s)		
	50 μm	100 μm	200 μm
NaF	1.63	6.5	26.1
Sucrose	2.34	9.4	37.5
Xylitol	3.05	12.2	48.9

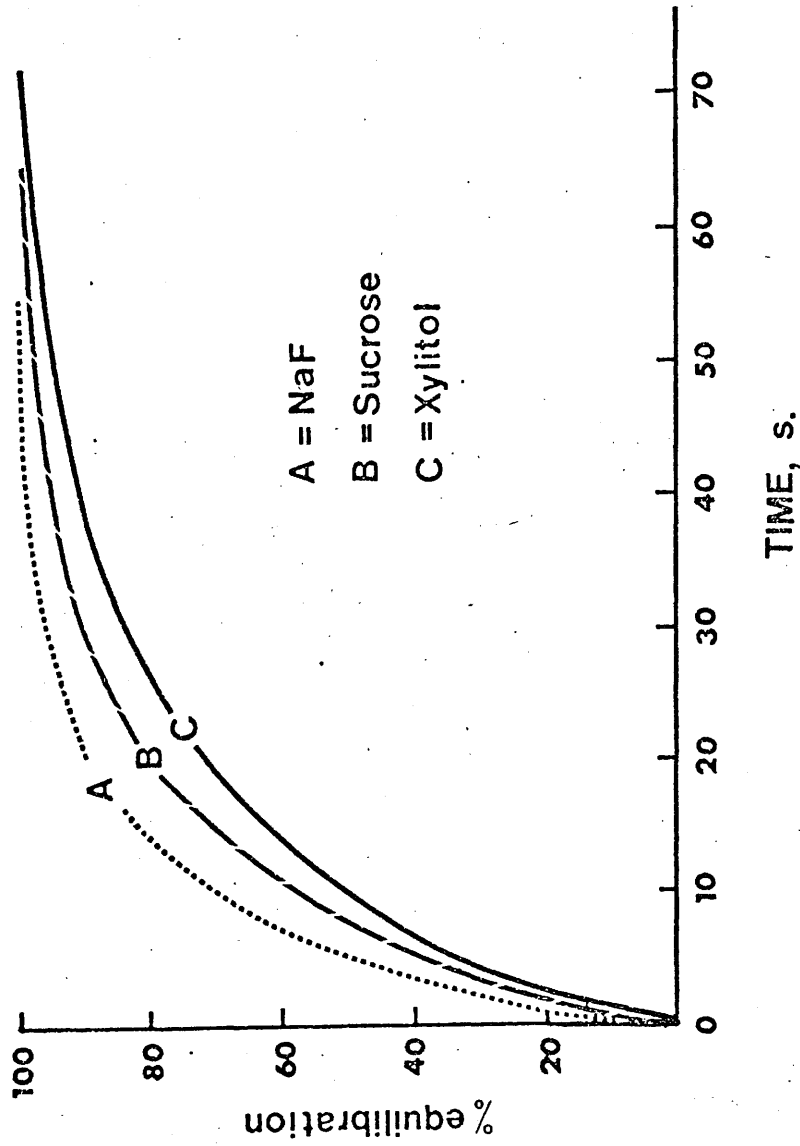


Fig. 5.2 Calculated rate of penetration of NaF, sucrose and xylitol into a 100 μm thick layer of plaque in the absence of metabolic or chemical interaction.

c is the concentration of sucrose in the outermost layer of plaque ($\mu\text{moles cm}^{-3}$), D is the diffusion coefficient of sucrose in plaque ($\text{cm}^2 \text{s}^{-1}$) and Q is the rate of metabolism of sucrose in plaque = F , the rate of flow of sucrose per unit volume of plaque ($\mu\text{moles cm}^{-3} \text{s}^{-1}$).

From Equation 3.5:-

$$F = \frac{2cD}{H^2} \mu\text{moles cm}^{-3} \text{s}^{-1} \quad \text{Equation 5.1}$$

The specific gravity of plaque is 1.04 g cm^{-3} (Darlington, 1979), so 1 mg of plaque occupies a volume of 0.00096 cm^3 . Thus, converting from cm^3 of plaque to mg of plaque, and from seconds to minutes, Equation 5.1 becomes:-

$$F = \frac{0.115cD}{H^2} \mu\text{moles mg}^{-1} \text{min}^{-1} \quad \text{Equation 5.2}$$

Equation 5.2 predicts that the maximum rate of flow of sucrose from saliva into plaque is directly proportional to the diffusion coefficient of sucrose in plaque and to the concentration of sucrose in saliva, and inversely proportional to the plaque thickness to the second power. The diffusion coefficient of sucrose in plaque was estimated to be $3.0 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$ (Table 5.3). From Equation 5.2 therefore, the maximum rate of flow of sucrose from saliva containing 100 mM sucrose (equivalent to about one teaspoonful of sugar in a cup of tea) into a layer of plaque 100 μm thick, is $0.35 \mu\text{moles mg}^{-1} \text{min}^{-1}$. This calculation assumes that the concentration of sucrose in the outermost layer of plaque equals the concentration of sucrose in saliva i.e. that the partition coefficient between plaque and saliva for sucrose is unity. This is unlikely to be correct because plaque contains large quantities of insoluble solids. If sucrose is confined to the extracellular fluid volume of plaque, the partition coefficient would be about 0.32, the

proportion of extracellular fluid in plaque (Edgar and Tatevossian, 1971). Thus, the calculated rate of flow of sucrose into plaque would be about three-fold slower. For accurate prediction of flow rates in plaque, the partition coefficient between plaque and saliva should be determined experimentally.

Listed in Table 5.5 are values of maximum rates of flow of sucrose from saliva into plaque, calculated using Equation 5.2, for plaque thicknesses of 25, 50, 100 and 200 μm with 100 mM sucrose in saliva, again assuming that the partition coefficient between plaque and saliva is unity. These values also apply to the situation where all of the sucrose is metabolised in the surface 25 to 200 μm of thicker layers of plaque. No direct measurements have been reported which would ascertain whether these flow rates would saturate the rates of sucrose metabolism within plaque in vivo i.e. acid production and polysaccharide synthesis. In a study by Darlington (1979), a subject, having accumulated plaque over a 24 h period, rinsed his mouth for 30 s with 10 ml of a 584 mM (20 per cent w/v) sucrose solution. At various times after the rinse, plaque was removed and centrifuged to separate the plaque fluid which was then assayed for sucrose. A minute or so after the end of the rinse the concentration of sucrose in the plaque fluid was still very high (301 mM) suggesting that the plaque had been saturated with sucrose. However, the interpretation of this experiment is difficult. A very high concentration of sucrose was used and the plaque thickness is unknown. In addition, some of the sucrose would diffuse out of the plaque during the interval between the end of the rinse and the commencement of plaque removal, and obviously some of the sucrose would have been lost by metabolism. The rate of penetration of

Table 5.5 Maximum rates of flow of sucrose from saliva into plaque layers of various thicknesses, when the sucrose concentration in saliva is 100 mM. Calculated using Equation 5.2 (see text for details).

Thickness of plaque layer (μm)	Maximum rate of flow of sucrose into plaque ($\mu\text{ moles mg}^{-1} \text{ min}^{-1}$)
25	5.52
50	1.38
100	0.35
200	0.086

sucrose into plaque illustrated in Fig. 5.2 applies also to clearance from plaque. For a 100 μm layer of plaque, clearance would be expected to be 80 per cent complete within 20 s, and 70 per cent complete within 1 min for a 200 μm layer. However this theory assumes that the concentration of sucrose in saliva remains zero (or very nearly so) during the clearance. Darlington (1979) also measured the concentration of sucrose in saliva following consumption of various foods and drinks such as sugared-tea, 'Coca-Cola', toffee and chocolate. Clearance of sucrose was not complete until between 3 and 8 min after the food or drink had been swallowed, and this would tend to maintain the presence of some sucrose in the plaque for the same period of time.

CHAPTER 6

RATES OF DIFFUSION OF ACIDS IN PLAQUE

6.1 INTRODUCTION

It was shown in Chapter 5 that sugars diffuse rapidly into dental plaque. Many workers have expressed the viewpoint that acids diffuse in plaque more slowly than sugars (see literature review, Section 2.3.1) despite a lack of corroborative experimental evidence. The aim of the work described in this chapter was to assess the rate of diffusion in plaque of some carboxylic acids, using the experimental technique already applied to the study of diffusion of NaF and sugars. Acetic and lactic acids were chosen for particular study because these are two of the acids found in highest concentrations in plaque after in vivo fermentation (Geddes, 1975; Gilmour et al., 1976).

Measurement of diffusion coefficients of acids presents practical difficulties, particularly because hydrogen ions are very reactive in the complex environment of dental plaque. Furthermore, dilute solutions of acetic and lactic acid may produce pH values of less than 4.0, which is outwith the physiological pH range of plaque and at which the acids are only partially dissociated. The use of strong buffers to bring the pH closer to neutrality might modify the rates of diffusion of acids by introducing high concentrations of other ions.

To circumvent these difficulties, it was decided to measure diffusion coefficients of sodium acetate and sodium lactate in plaque by monitoring the clearance from plaque of acetate and lactate ions. In aqueous solution, these salts produce a pH close to neutrality and diffuse only about 13 per cent more slowly than the acids (see Appendix). Therefore, measurement of the diffusion coefficients of sodium acetate and sodium lactate in plaque should provide a reliable estimate of the rate of diffusion of carboxylic acids in plaque. As a further precaution, it was found to be necessary to prevent metabolic activity within the plaque during the diffusion experiment, and this was again achieved by fixation of the plaque with glutaraldehyde.

6.2 METHODS

6.2.1 Experimental Technique

The experimental technique adopted to measure diffusion coefficients of sodium acetate and sodium lactate in plaque at 37°C, was similar to that used for NaF (Section 4.2) and for sucrose (Section 5.2). Five young adult subjects (SM, DW, HMA, LJ and LS) accumulated plaque for this study by refraining from toothcleaning for periods of 24 h. Plaque was collected as described in Section 3.3.1 and tested individually. The plaque sample was layered into the brass dish and retained in position with a Millipore filter and a wire frame as in Fig. 4.1. The plaque was then fixed by immersing this assembly for 1.5 h in 5 ml of a 2.5 per cent solution of glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer, pH 7.2, at room temperature.

For acetate diffusion experiments, the assembly was transferred for a 20 min equilibration phase in 10 ml of a 200 mM solution of sodium acetate in 100 mM glycine - NaOH buffer at 37°C. The buffer pH, initially 7.0 changed to 7.4 on addition of the acetate. Clearance of acetate from the plaque was then monitored by suspending the plaque assembly for 12 min in 2 ml of the 100 mM glycine -NaOH buffer (pH 7.0) at 37°C and sampling 25 µl aliquots from the buffer solution after 2, 3, 4, 5, 6, 8, 10 and 12 min. The equilibration and clearance solutions were continuously stirred using magnetic stirrers. The 25 µl aliquots, and the plaque and filter after the 12 min clearance, were stored on ice for acetate assay by isotachopheresis (details to follow in Section 6.2.2).

The experimental technique for measuring the diffusion coefficient of sodium lactate was very similar. The equilibration solution was a 300 mM solution of sodium lactate in 100 mM glycine - NaOH buffer. The buffer pH, initially 7.0, changed to 6.8 on addition of the lactate. The glycine - NaOH buffer with no added lactate was used as the clearance solution. Assay of lactate was also by isotachopheresis. The reagents used for these experiments were AnalaR grade (B.D.H. Ltd.) except the sodium lactate which was laboratory grade (B.D.H. Ltd.).

Clearance curves were constructed from the acetate and lactate assays by plotting the amount of acetate or lactate within the plaque on a logarithmic scale against time. As the theory outlined in Section 3.2 is expected to apply to these experiments (i.e. that the clearance curve should be single exponential)

regression analysis was used to calculate a value for the clearance halving-time, $T_{\frac{1}{2}}$, and the diffusion coefficient was calculated using Equation 3.4.

6.2.2 Acetate and Lactate Assay

The measurement of acetate and lactate was performed using the technique of isotachopheresis on an LKB 2127 Tachophor (LKB Produkter AB, Bromma, Sweden). Isotachopheresis is an electrophoretic technique for separating ions according to their different mobilities in an electric field. Upon separation, the ions migrate with the same velocity in consecutive zones between selected leading and terminating electrolytes, producing a constant electric current. The zones are differentiated either thermally, using a thermocouple to detect the slightly different temperatures of the zones, or by measuring the different ultra-violet absorbances of the zones. The zone length is proportional to the amount of ion present and calibration is performed by injecting standard solutions of the ions under study. Isotachopheresis generally does not require pretreatment of the samples and is suitable for rapid analysis of samples of volume as small as 0.5 μ l and can detect less than 1 nmole of substance. The theory, instrumentation and applications of isotachopheresis are comprehensively reviewed by Everaerts, Beckers and Verheggen (1976).

For this study, acetate and lactate ions were measured using a technique similar to that described by van der Hoeven et al. (1978). The separation was performed in a 61 cm, two-turn capillary (LKB Part No. 2127-124) operated at 23°C with a constant current of 50 μ A. The leading electrolyte was 10 mM HCl buffered to pH 4.0

with β -alanine and containing 0.1 per cent Triton X-100. The terminating electrolyte was 5 mM octanoic (or caprylic) acid. Using standard solutions of acids, the ions in each sample were identified both thermally and by U.V. absorbance measurements. With this technique it is possible to separate and quantify formate, pyruvate, phosphate, lactate, succinate, acetate and propionate ions.

Of the 25 μ l aliquot samples from the clearance solutions, 20 μ l were injected into the capillary, without pretreatment. Plaque, usually about 11 mg, was transferred from the brass dish to a 0.75 ml polypropylene tube (Walter Sarstedt U.K. Ltd., Leicester, England, Catalogue No. 30/8) and weighed to 0.01 mg on a Mettler balance (Type H16). The amount of plaque remaining in the dish, usually about 1 mg, was also weighed. Two hundred μ l of leading electrolyte buffer without Triton X-100 were added to the tube and mixed with the plaque for 30 s on a vortex mixer. The tube was then centrifuged at 10,000 g for 15 min at 4^oC on a MSE High Speed 18 Centrifuge (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England). From the amount of acetate or lactate in 20 μ l of the supernatant injected into the capillary, the total amount of acetate or lactate in the plaque was calculated, making allowance for the amount of plaque left in the brass dish. A similar technique was used to measure the acetate or lactate in the filter, except that the filter was mixed with 50 μ l of buffer. The lowest amount of acetate detected for any of these samples was about 10 nmoles in a volume of 20 μ l (a concentration of 0.7 μ moles ml⁻¹), with a measurement precision of about 5 per cent.

All of the reagents used in these analyses were obtained from B.D.H. Ltd. The HCl was AnalaR grade, the β -alanine was chromatographically pure, and the octanoic acid and Triton X-100 were laboratory grade.

6.3 RESULTS

A typical clearance curve for sodium acetate is shown in Fig. 6.1. Six samples for acetate analysis and eight samples for lactate analysis were lost due to bubble formation within the capillary, so some of the clearance curves were constructed from six or seven data points, instead of eight (Tables 6.1 and 6.2). However, all of the clearance curves were single exponential and the regression analysis revealed highly significant correlations ($r \geq 0.93$, $p < 0.001$, except for one curve with six data points, for which $p < 0.01$). The results of six measurements of diffusion coefficients of sodium acetate and of sodium lactate in plaque, are summarised in Tables 6.1 and 6.2, respectively.

The mean diffusion coefficient of sodium acetate in glutaraldehyde-fixed plaque at 37°C , \pm standard deviation, was $(5.01 \pm 0.73) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, and for sodium lactate was $(4.78 \pm 0.30) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$.

At the end of the clearance phase, the pH of the glycine buffer was 7.2 for the acetate experiments, and 7.0 for the lactate experiments. Therefore, for acetate, equilibration was at pH 7.4 and clearance was into buffer at pH 7.0 rising to 7.2. For lactate, equilibration was at pH 6.8 and clearance was into buffer of constant

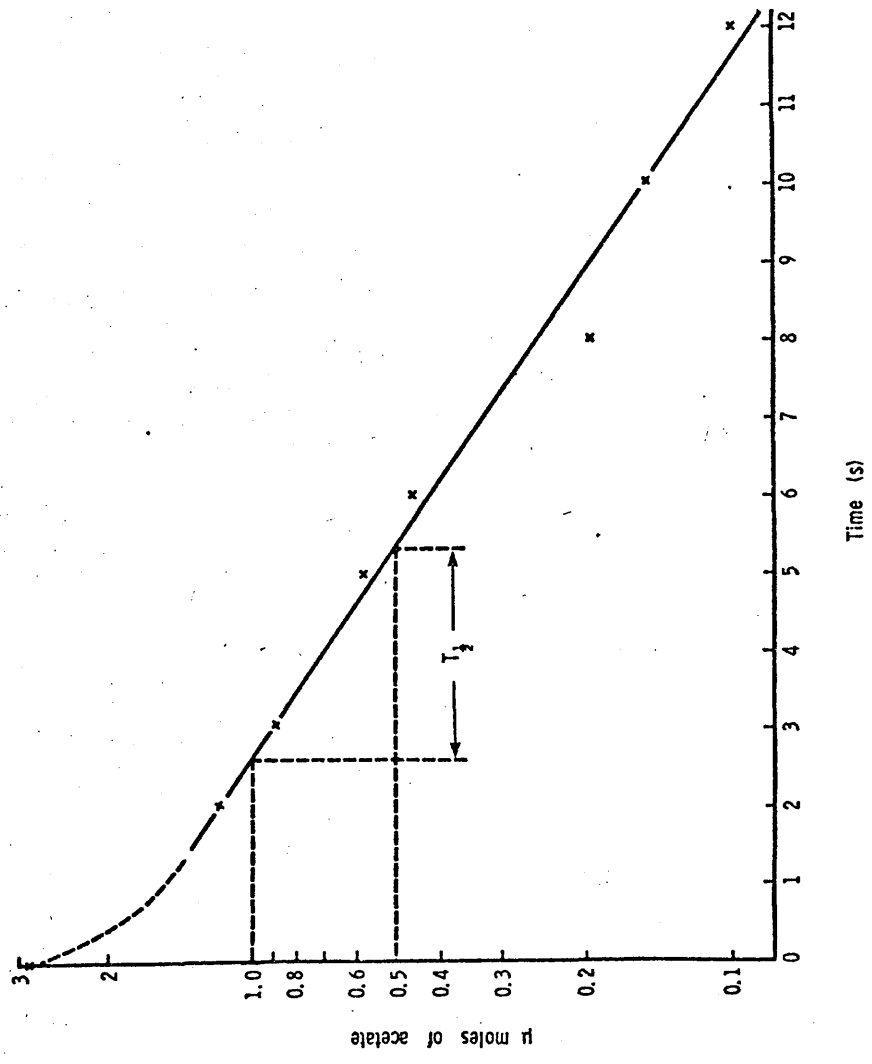


Fig. 6.1 Clearance of acetate from a glutaraldehyde-fixed plaque sample from subject DW (1st measurement for DW reported in Table 6.1).

Table 6.1 Measurement of the diffusion coefficient of sodium acetate in glutaraldehyde-fixed plaque at 37°C.

r = correlation coefficient; n = number of data points;

$T_{\frac{1}{2}}$ = clearance halving-time; D = diffusion coefficient

Subject	r	n	$T_{\frac{1}{2}}$ (s)	D ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
SM	0.980	7	208	4.74
DW	0.992	7	164	6.02
DW	0.993	7	175	5.65
HMA	0.976	7	250	3.95
HMA	0.936	8	201	4.92
LS	0.988	6	208	4.76

mean D, \pm standard deviation = $(5.01 \pm 0.73) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$

Table 6.2 Measurement of the diffusion coefficient of sodium lactate in glutaraldehyde-fixed plaque at 37°C.

r = correlation coefficient; n = number of data points;

$T_{\frac{1}{2}}$ = clearance halving-time; D = diffusion coefficient

Subject	r	n	$T_{\frac{1}{2}}$ (s)	D ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
HMA	0.937	8	201	4.91
HMA	0.938	6	216	4.57
SM	0.998	7	220	4.49
DW	0.991	6	204	4.84
DW	0.994	6	216	4.58
LJ	0.996	7	187	5.29

mean D, \pm standard deviation = $(4.78 \pm 0.30) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$

pH of 7.0.

6.4 DISCUSSION

The diffusion coefficient of sodium acetate in glutaraldehyde-fixed plaque at 37°C ($5.01 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) is lower, by a factor of 3.2, than the diffusion coefficient of sodium acetate in water at 37°C ($1.61 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$; see Appendix). The diffusion coefficient of sodium lactate in fixed plaque ($4.78 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) is 4.6 per cent lower than for sodium acetate, but this difference is not of statistical significance ($t = 0.714$, $p > 0.4$, Student's t -test). For sodium lactate, diffusion in fixed plaque is also slower by a factor of 3.2 than diffusion in water (diffusion coefficient of sodium lactate in water at 37°C = $1.55 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$; see Appendix). If it is assumed that, as for NaF, sucrose and xylitol, the effect of glutaraldehyde-fixation upon rates of diffusion in plaque is small, then it is clear that sodium acetate and sodium lactate diffuse rapidly in plaque. For a 100 μm plaque layer, the clearance-halving times would be 5.61 and 5.88 s for the acetate and lactate, respectively. Thus, 90 per cent equilibration, whether by penetration or clearance, would be achieved within 20 s.

Acetic and lactic acids are expected to diffuse faster than the sodium salts because the hydrogen ion has a higher mobility than the sodium ion. However, in water, acetic acid diffuses only 13 per cent faster than sodium acetate (see Appendix). If this factor is applicable to plaque, the diffusion coefficients of acetic

and lactic acids in plaque at 37°C would be 5.7×10^{-6} and 5.4×10^{-6} $\text{cm}^2 \text{s}^{-1}$, respectively.

These values are consistent with those reported by Dibdin (1979) who measured diffusion coefficients of carboxylic acids in plaque using a diaphragm diffusion cell technique. Dibdin also found these acids to diffuse slightly faster below their pK_a 's, where a high proportion of the acid is undissociated. Using a penetration profile study, Tatevossian (1979), measured diffusion coefficients of sodium ^{14}C -acetate and sodium ^{14}C -lactate in water at 37°C to be 1.06×10^{-5} and $1.01 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, respectively. These values are about 30 per cent lower than previously published values (see Appendix) although the standard deviations quoted by Tatevossian are high (86 per cent for acetate, 25 per cent for lactate). Tatevossian further estimated the diffusion coefficients of sodium ^{14}C -acetate and sodium ^{14}C -lactate to be 3.24×10^{-6} and $3.38 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, respectively, for plaque fluid, and 1.29×10^{-6} and $1.42 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, respectively, for plaque sediment. These values are likely to be erroneously low because of metabolic activity within the plaque and binding of the diffusing ions to plaque constituents. Tatevossian's measurement of the amount of ^{14}C in successive layers into the sample does not differentiate between the ions which are mobile and those which are bound. The experimental techniques and the results reported by Dibdin and by Tatevossian are discussed in more detail in Chapter 10.

By monitoring clearance of acetate and lactate ions from plaque, the effect of binding of these ions to plaque constituents

is largely circumvented because strongly bound ions will not be detected and will play no part in the diffusion process. There is likely to be some residual metabolically-formed acetate and lactate in the plaque (Geddes, 1975; Gilmour et al., 1976). These ions will be free to diffuse with the acetate and lactate ions of the equilibration solution. However, if acetate and lactate are produced during the clearance phase of the experiment, the mathematical theory outlined in Section 3.2 will no longer apply, and any estimate of the diffusion coefficient will be in error. For this reason it was essential to prevent acid production within the plaque using glutaraldehyde, as in Chapter 5.

The results reported in Tables 6.1 and 6.2 and in Chapter 4 show that in plaque, sodium acetate and sodium lactate diffuse about 15 per cent faster than NaF. This difference is shown to be of statistical significance using Student's t-test (for acetate, $t = 2.433$, $0.025 > p > 0.01$; for lactate, $t = 3.326$, $0.005 > p > 0.001$). This result is surprising because in water NaF diffuses about 18 per cent faster than sodium acetate or sodium lactate. It has been shown in this thesis that the ratio of the diffusion coefficient in water to the diffusion coefficient in plaque is 2.15 for xenon, 2.33 for sucrose, 3.2 for sodium acetate and sodium lactate, and 4.4 for NaF. The rate of diffusion in plaque of charged species is reduced by a relatively greater amount than for uncharged species. The large reduction in the rate of diffusion of NaF may be related to the high charge density of the fluoride ion.

The results also show that in plaque, sodium acetate and

sodium lactate diffuse faster than sucrose. This difference is statistically significant according to Student's t-test (for acetate, $t = 6.024$, $p < 0.001$; for lactate, $t = 9.192$, $p < 0.001$). If acids diffuse faster in plaque than sugars, why is there a build-up of acid in plaque following a sugar mouthrinse, as shown by Stephan curves? This is a complex question to answer. There are at least three principal parameters to consider: the rate of diffusion of sugar into plaque, the rate of acid production within plaque and the rate of diffusion of acids from plaque. The two rates of diffusion are now known and the following analysis may reveal how these three parameters combine.

It will be assumed that acid is being produced in a layer of plaque at a constant rate, a (arbitrary units s^{-1}). Acid will diffuse from the layer in an exponential manner with a time constant b (s^{-1}). If the amount of acid in the plaque at any given time is N_1 (arbitrary units) then the rate of clearance of acid from the plaque will be bN_1 (arbitrary units s^{-1}). Thus, the net change in the amount of acid in the plaque, dN_1 , in a short time interval dt , is given by the following expression:-

$$\frac{dN_1}{dt} = a - bN_1$$

Equation 6.1

$$\text{or } dN_1 + bN_1 dt = a dt$$

Multiplying both sides by e^{bt} and integrating:-

$$\int e^{bt} dN_1 + \int bN_1 e^{bt} dt = \int a e^{bt} dt \quad \text{Equation 6.2}$$

$$\begin{aligned} \text{Now, } \frac{d}{dt} (N_1 e^{bt}) &= N_1 \frac{d}{dt} (e^{bt}) + e^{bt} \frac{d}{dt} (N_1) \\ &= bN_1 e^{bt} + e^{bt} \frac{dN_1}{dt} \end{aligned}$$

Therefore, Equation 6.2 becomes:-

$$N_1 e^{bt} = \frac{a}{b} e^{bt} + c \quad \text{Equation 6.3}$$

where c is a constant.

If the amount of acid in the plaque at $t = 0$ is N_0 , then from Equation 6.3,

$$\begin{aligned} N_1 &= \frac{a}{b} + c = N_0 \\ \Rightarrow c &= N_0 - \frac{a}{b} \end{aligned}$$

Thus, Equation 6.3 becomes:-

$$\begin{aligned} N_1 e^{bt} &= \frac{a}{b} e^{bt} + N_0 - \frac{a}{b} \\ \Rightarrow N_1 &= \frac{a}{b} + N_0 e^{-bt} - \frac{a}{b} e^{-bt} \\ \Rightarrow N_1 &= N_0 e^{-bt} + \frac{a}{b} (1 - e^{-bt}) \quad \text{Equation 6.4} \end{aligned}$$

N_1 is shown plotted against t in Fig. 6.2 for a range of values of a and b and $N_0 = 1$. If $a = 0$, the second term in Equation 6.4 becomes zero leaving only the exponential clearance term.

N_1 therefore falls quickly to zero as the acid diffuses from the plaque, at a rate determined by the value of b . This is

illustrated in Fig. 6.2 for $a = 0$, $b = 1$. If $b = 0$, it can be

shown by MacLaurin expansion of $(1 - e^{-bt})$ that Equation 6.4

reduces to $N_1 = N_0 + at$ i.e. N_1 increases linearly with time

according to the value of a . This is illustrated in Fig. 6.2 for

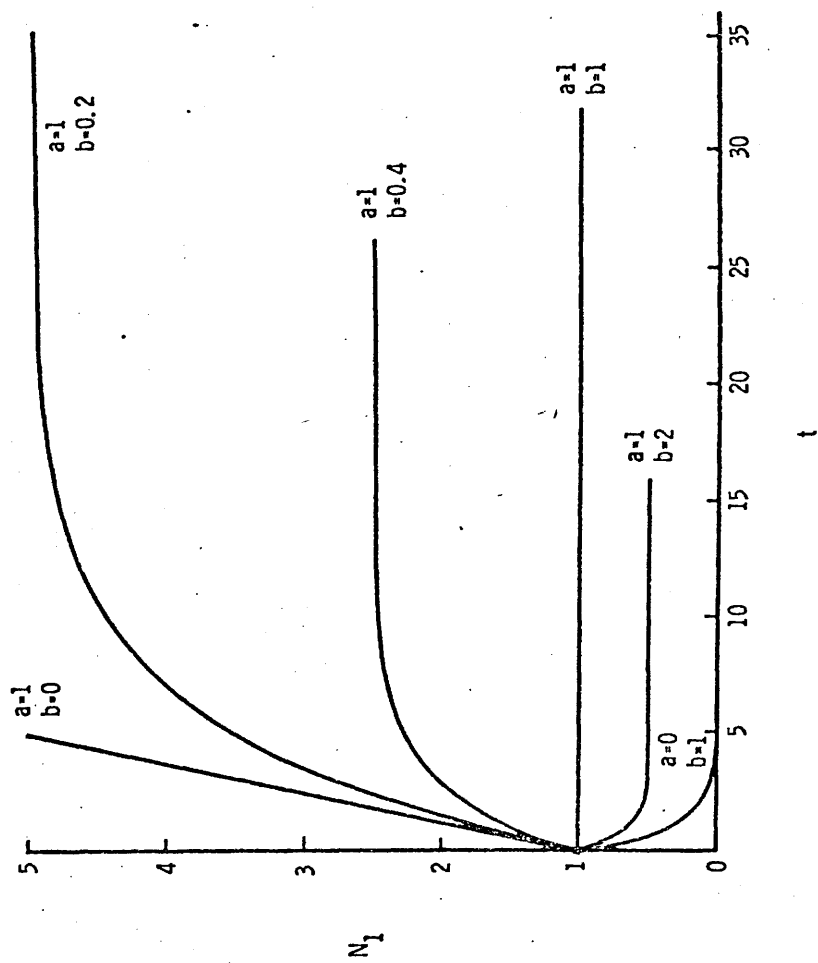


Fig. 6.2 Plot of $N_1 = N_0 e^{-bt} + \frac{a}{b} (1 - e^{-bt})$ against t for $N_0 = 1$ and various values of a and b .

$a = 1, b = 0$. For the special case $a = 1$ and $b = 1$, N_1 remains constant.

The other curves in Fig. 6.2 show that after a time of approximately $5/b$, N_1 reaches a constant value at which $N_1 b = a$ i.e. the rate of acid production = rate of clearance of acid by diffusion. This can also be deduced mathematically from Equation 6.4 when t becomes large.

From Equation 3.2, in the absence of acid production in the plaque,

$$N_1 = N_0 e^{-\left[D \pi^2 t / 4L^2 \right]}$$

where L is the thickness of the plaque layer.

Thus:-
$$b = \frac{D \pi^2}{4L^2}$$

or
$$b = \frac{2.47 D}{L^2} \text{ s}^{-1} \quad \text{Equation 6.5}$$

Therefore, if $N_1 b = a$,

$$N_1 = \frac{aL^2}{2.47 D} \quad \text{Equation 6.6}$$

Thus the equilibrium level of acid in the plaque is directly proportional to the rate of acid production and inversely proportional to the rate of diffusion. The faster the diffusion rate, the lower will be the acid level, but the acid level will not reach zero unless the rate of acid production is zero. This will happen in vivo when the carbohydrate source, including stores of intracellular polysaccharide, is depleted. Equation 6.6 also states that the plaque acid concentration is proportional to the plaque

thickness to the second power, so that by doubling the plaque thickness, the equilibrium acid concentration would increase four-fold, assuming no substrate limitation.

Strålfors (1950) derived an expression similar to Equation 6.6, to describe the acid concentration in plaque adjacent to the enamel surface. The constant of proportionality in the denominator was 2 instead of 2.47. Strålfors also performed a series of laboratory model experiments to illustrate this theory, in which a glass pH electrode was coated with bacteria-containing agar gel and exposed to sugar solutions. The acid concentration at the inner gel surface was shown to increase with the bacterial concentration, which in turn was proportional to the acid production rate, and also increased with gel thickness.

The diffusion coefficient of acetic and lactic acids in dental plaque has been estimated in this chapter to be about $5.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. For a plaque layer of thickness 100 μm , Equation 6.6 therefore predicts an equilibrium acid concentration $N_1 = 7.36a$. It was estimated in Chapter 5 that the maximum rate of flow of sucrose into a 100 μm layer of plaque, from saliva containing 100 mM sucrose, is 0.35 μmoles of sucrose per mg plaque per min or $5.8 \text{ nmoles mg}^{-1} \text{ s}^{-1}$. If plaque is capable of converting sucrose to acid at this rate, then, allowing for four moles of acid per mole of sucrose, the equilibrium acid concentration in the plaque would be $171 \text{ nmoles mg}^{-1}$. Taking the specific gravity of plaque to be 1.04 g cm^{-3} (Darlington, 1979) this is equivalent to a concentration of 164 mM.

This concentration is within the observed physiological range for plaque in vivo. Geddes (1975) reported a total acid concentration in 24 h plaque of about 50 mM, measured 5 min after a 1 min exposure to 40 per cent sucrose (1.17 M). Gilmour et al. (1976) reported acid concentrations of up to 200 mM for 7-day plaque, measured 4 min after a 30 s rinse with 20 per cent sucrose solution.

The above analysis is a simplified version of what happens in the mouth. No allowance has been made for the partition coefficient between plaque and saliva for sucrose (see Section 5.4) which, if numerically less than unity, would lead to a lower estimate of the rate of penetration of sucrose into plaque and hence also of the rate of acid production and the equilibrium acid concentration. Furthermore, no allowance has been made for polysaccharide production, the different types of acid produced, inhibition of acid production at low pH, the possible variation of metabolic activity at different depths in the plaque, or for buffering within the plaque and from saliva. As shown in Table 5.3, the maximum rate of penetration of sucrose into plaque is proportional to the concentration of sucrose in saliva and inversely proportional to the square of the thickness of the plaque layer. It seems likely that for thin layers of plaque, the rate of sucrose penetration may saturate the rate of metabolism, so that the plaque acid concentration will be determined by the rate of acid production and the rate of diffusion of acids from the plaque. For thicker layers of plaque, the rate of acid production, and hence the plaque acid concentration, may be limited by the rate of sucrose penetration.

The analysis clearly shows that the rapid rate of diffusion of acids from plaque limits, rather than causes a build-up, of the acid concentration in plaque. In the example above, if the diffusion coefficients of acids in plaque were the same as the diffusion coefficient for sucrose in plaque, then the predicted plaque acid concentration would be 301 mM instead of 164 mM. In the absence of continued metabolism, 90 per cent of the acid would diffuse from a 100 μm plaque layer within 20 s. Measurements of Stephan curves have shown that following a sucrose mouthrinse, it may be 30 min or more before the plaque pH returns to resting levels. It would seem that this delay is caused, not by slow rates of diffusion, but by continued metabolism within the plaque of residual sucrose and of polysaccharide stores.

CHAPTER 7

ASSESSMENT OF THE INFLUENCE OF GLUCAN ON DIFFUSION

OF NaF AND SUCROSE

7.1 INTRODUCTION

König (1966) first postulated the theory that slimy extracellular polysaccharides act as a barrier to diffusion in dental plaque. As has been discussed in detail (Section 2.3), this concept was perpetuated by several workers, in particular by Winter (1968), Kleinberg (1970), Guggenheim, Regolati and Mühlemann (1972), Cole (1977) and Bowen (1976, 1978). The only experimental evidence to support this postulate is a study by Hojo, Higuchi and Araya (1976) who showed that, when grown on a glass pH electrode, a strain of Streptococcus mutans PK1 which synthesised large amounts of insoluble glucan, maintained lower pH values than a second strain which synthesised small amounts of insoluble glucan. However, the authors failed to prove that this effect was caused by different rates of diffusion and the relative rates of acid production by the two strains was not reported.

The aim of the work described in this chapter was to investigate the effect of glucan on diffusion using the microorganism Streptococcus sanguis 804 (NCTC 10904), an isolate from dental plaque (Carlsson, 1965). It has been shown (Carlsson, 1968; Beeley and Black, 1977) that S. sanguis 804 produces extracellular glucosyltransferase when grown in batch culture with glucose as the

carbohydrate source. This enzyme can polymerise the glucose moiety of sucrose to form high molecular weight dextran-like glucans, similar to those which form the plaque matrix. S. sanguis is further suited to this study as it is one of the earliest organisms to colonise the tooth surface in plaque formation (Ørstavik, Kraus and Henshaw, 1974).

Glucans are produced from sucrose but not from glucose, therefore S. sanguis 804 was grown in batch culture initially with glucose as the sole carbohydrate source to produce a glucan-free culture. Sucrose was then added to produce a glucan-containing culture. Diffusion coefficients were measured for NaF and ^{14}C -sucrose in glucan-free and glucan-containing bacterial sediments and in bacterial-free glucan samples from these cultures.

7.2 MATERIALS AND METHODS

7.2.1 Growth of Streptococcus Sanguis 804 (NCTC 10904) in Batch Culture

The Streptococcus sanguis 804 (NCTC 10904) used in this experiment was a gift from Professor J. Carlsson, University of Umeå, Uppsala, Sweden to Dr J.A. Beeley, Department of Oral Biochemistry, University of Glasgow Dental Hospital and School. The bacteria were maintained aerobically on blood agar at 4°C and subcultured at monthly intervals.

S. sanguis 804 was grown in batch culture using the

apparatus described by Beeley and Black (1977) and which is illustrated in Fig. 7.1. To a culture flask containing 750 ml of Carlsson's diffusate medium (Carlsson, Newbrun and Krasse, 1969) with one per cent (w/v) glucose, was added a 12.5 ml inoculum of a 16 h culture of S. sanguis 804 in Carlsson's diffusate medium. The culture was continuously stirred with a magnetic stirrer, while being incubated aerobically at 37°C in a water bath with a thermostat-controlled immersion heater. The pH of the culture was maintained at 7.0 using a steam-autoclavable combination electrode (Activion Glass Ltd., Kinglassie, Fife, Scotland) linked to a Radiometer titrator, type TTT1 (Radiometer A/S, DK 2400 Copenhagen NV, Denmark), which regulated the addition of 3M NaOH to the culture by a peristaltic pump. A chart recorder (Radiometer Titrigraph SBR1c) monitored the cumulative amount of NaOH added to the culture. Prior to starting the culture, the medium, alkali and glassware were steam autoclaved for 20 min at 103.4 kPa and 121°C.

The culture was inoculated at 5.00 pm and at 9.00 am the following morning the culture medium was turbid. The chart recorder trace indicated that there had been a phase of alkali addition to the culture but during the final 2-3 h the rate of alkali addition had decreased and practically ceased. Approximately 60 ml of the culture medium was siphoned off into two tubes and centrifuged at 5000 g for 15 min at 4°C in a Sorvall RC-5 Superspeed Centrifuge (Sorvall/Du Pont Instruments, Newtown, Connecticut, U.S.A.). The supernatants were poured off and the bacterial sediments were twice washed by resuspending in 0.85 per cent NaCl, shaking on a vortex mixer for about 30 s, and

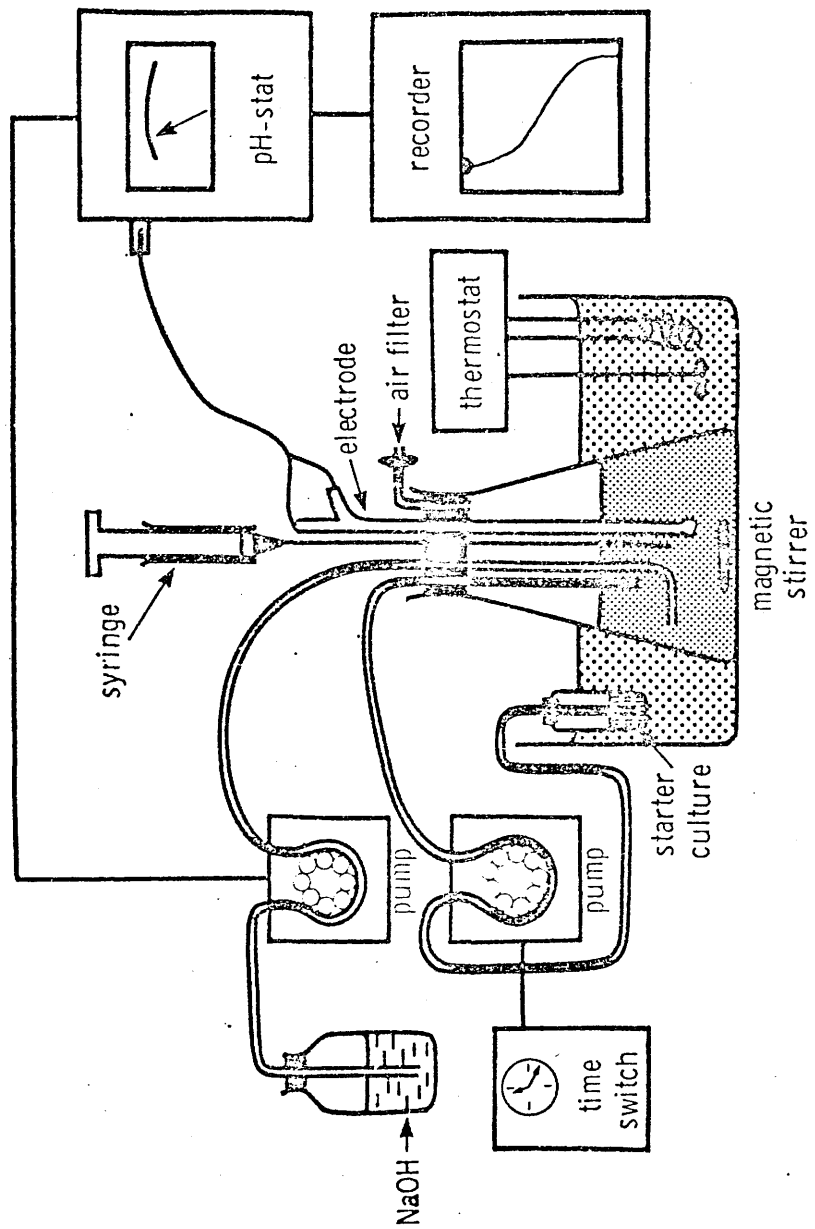


Fig. 7.1 Batch culture apparatus used for growth of Streptococcus sanguis 804 (NCIC 10904).

re-centrifuging. One of these samples of glucan-free bacterial sediment was analysed for total carbohydrate and DNA content (details to follow), and the other used for diffusion studies.

Meanwhile, a 25 per cent (w/v) aqueous solution of sucrose was added aseptically to the culture flask in sufficient quantity to produce a five per cent (w/v) solution of sucrose in the culture medium. This resulted in further alkali intake by the culture, and further bacterial samples were harvested at various times between 1.25 and 23 h after sucrose addition. These glucan-containing samples were centrifuged and washed as before. For all cultures, alkali addition had practically ceased after 23 h of growth on sucrose. The purity of the culture was assessed by examining Gram stains of the bacterial samples.

Culture supernatant at the end of the glucose phase of one culture was retained and further centrifuged at 10,000 g for 1 h at 4°C. The resultant supernatant, containing glucosyltransferase but few or no bacterial cells, was divided into three aliquots and diluted 1:1 with a 10 per cent (w/v) solution of sucrose in 0.1 M sodium phosphate buffer, pH 7, containing 0.02 per cent (w/v) of the bactericide sodium azide. These mixtures were incubated overnight at 37°C, producing a slimy glucan-like material. This material was centrifuged at 5000 g for 15 min at 4°C with two washings with 0.85 per cent NaCl (as for the bacterial samples). The rate of diffusion of NaF in these cell-free glucan samples was then measured.

7.2.2 Measurement of Total Carbohydrate in Bacterial and Glucan Samples

The bacterial and glucan samples were dried to constant weight over phosphorus pentoxide in a vacuum flask. Samples were weighed before and after drying, to 0.01 mg on a Mettler balance (Type H 16). About three days were required for complete drying, with a yield of 30 mg or more (dry weight) from 30 ml of culture medium. This sample was dissolved by adding 10 N NaOH in the ratio of 3 μ l per mg, and the total volume made up to 25 ml with double-distilled, de-ionised water. Part of this solution was used for carbohydrate analysis and part was retained for DNA analysis.

Total carbohydrate analysis was performed using P.J. Somers' modification (personal communication) of the cysteine-sulphuric acid colorimetric method first described by Dische, Shettles and Osnos (1949). Serial dilutions were made of the sample solutions described above (dilution factors ranged from five to 200). Standard aqueous carbohydrate solutions were prepared using glucose, to cover the concentration range 0-50 μ g ml⁻¹. Immediately prior to the analyses, the reactive agent was prepared by adding 0.5 ml of a 3 per cent aqueous solution of L-cysteine hydrochloride to 25 ml of an 86 per cent aqueous solution of sulphuric acid placed in an ice bath. 0.5 ml of this solution was added to 0.1 ml of each of the standard and sample solutions, again on ice. The tubes were shaken and heated for exactly 3 min in a water bath at 100°C, during which a yellow colour developed, and then immediately plunged into ice-water. The absorbances of these solutions were measured at 420 nm using a Unicam SP 500 series 2 Spectrophotometer (Pye Unicam Instruments Ltd., Cambridge, England).

The assays were performed at least four times for the bacterial samples and in duplicate for standards. Dilution factors for samples were chosen to yield carbohydrate concentrations close to $25 \mu\text{g ml}^{-1}$ for optimum precision.

The chemicals used in this assay were AnalaR grade (B.D.H. Ltd.) except the L-cysteine hydrochloride (Sigma London Chemical Company Ltd., Poole, Dorset, England). Aqueous solutions were prepared using double-distilled, de-ionised water.

7.2.3 Measurement of Total DNA in Bacterial and Glucan Samples

The total DNA content of the bacterial and glucan samples was assayed using the spectrophotometric technique described by Burton (1956). DNA was extracted by adding 0.25 ml of 1 N perchloric acid to 0.25 ml of the sample solutions described in Section 7.2.2, and heating at 70°C for 15 min. Standard solutions prepared from sodium-DNA to cover the concentration range 0 to $75 \mu\text{g DNA ml}^{-1}$, were treated in the same way. Diphenylamine (DPA) reagent was prepared by dissolving 1.5 g of DPA in 100 ml of glacial acetic acid and adding 1.5 ml of concentrated sulphuric acid. Immediately prior to the analysis, 0.5 ml of a 1.6 per cent aqueous solution of acetaldehyde was added to 100 ml of the DPA reagent. One ml of this acetaldehyde-DPA reagent was added to each of the sample and standard solutions (0.5 ml) and these were incubated for 17 h at 30°C , producing a blue colour. The absorbances of these solutions were then measured at 600 nm using the Unicam SP 500 Spectrophotometer. Standards were assayed in duplicate and samples at least four times.

The chemicals used in this assay were AnalaR grade (B.D.H. Ltd.) with the exception of acetaldehyde (laboratory grade, B.D.H. Ltd.) and sodium-DNA salt (Type III, Sigma Ltd.). Double-distilled, de-ionised water was again used to prepare aqueous solutions.

7.2.4 Measurement of the Diffusion Coefficient of NaF in Bacterial and Glucan Samples.

The diffusion coefficient of NaF in the bacterial and glucan samples was measured at 37°C using the technique described for plaque (Section 4.2). About 12 mg of the bacterial or glucan sediment was layered into a 0.57 mm deep well in a brash dish and a thin Millipore filter (25 µm thick, 0.45 µm pore diameter) was retained over the plaque with a stainless-steel wire frame (Fig. 4.1). This assembly was immersed in 10 ml of a 2.2 per cent solution of NaF in 7 mM potassium phosphate buffer, pH 6.8 (resultant pH 6.6). After 12 min equilibration, the assembly was transferred to 10 ml of the buffer with no added NaF, and the clearance of fluoride from the sample was monitored by removing 0.1 ml aliquots from the buffer solution each minute for 7 min. Orion fluoride-specific electrodes were used to measure the amount of fluoride in these aliquots and in the sample and filter at 7 min and a clearance curve was plotted i.e. a graph of the amount of fluoride remaining in the sample versus time. By fitting an exponential to the data using the method of least squares fit, the clearance halving-time was calculated, and the diffusion coefficient was then calculated using Equation 3.4.

7.2.5 Measurement of the Diffusion Coefficient of ^{14}C -Sucrose in Bacterial Samples.

The diffusion coefficient of ^{14}C -sucrose in the bacterial samples was measured at 37°C using the technique described for plaque (Section 5.2). Bacterial sediment was supported in the brass dish as before, and the sample was fixed for 1.5 h by suspension in 5 ml of a 2.5 per cent solution of glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer, pH 7.2. This fixation was intended to circumvent metabolism of sucrose by the sample during the experiment. The sample was equilibrated for 40 min in 10 ml of 7 mM potassium phosphate buffer (pH 6.8) containing approximately 15 μCi of ^{14}C -sucrose, made up to a concentration of 100 mM with non-labelled sucrose. Clearance of ^{14}C -sucrose from the sample was monitored by suspending the assembly in 10 ml of sucrose-free buffer and sampling 0.1 ml aliquots over a 20 min period. The amount of ^{14}C -sucrose in these aliquots and in the bacterial sample and filter at 20 min was determined by liquid scintillation counting and a clearance curve was plotted as for the NaF experiments. An exponential was fitted by the method of least squares, to yield a value for the halving-time, and the ^{14}C -sucrose diffusion coefficient was calculated using Equation 3.4. For details of the chemicals used in these experiments, see Section 5.2.

As a control experiment, diffusion coefficients of NaF in glutaraldehyde-fixed bacterial sediments from the same cultures were measured. Due to the strict time schedule for sampling from the cultures and centrifuging, there was insufficient time to perform these control experiments concurrently with the ^{14}C -sucrose experiments. Accordingly, about half of each of the

bacterial sediments was immersed in 5 ml of the glutaraldehyde fixative and stored at room temperature for one or two days. The samples were then sectioned using a scalpel and fitted into the brass dish and the control experiments were performed as in Section 7.2.4 for diffusion of NaF in non-fixed samples.

7.3 RESULTS

7.3.1 Growth of Bacteria

About two hours after inoculation of S. sanguis 804 into glucose-containing Carlsson's medium, the chart recorder tracing indicated a period of alkali addition to the culture which lasted about 10 hours. During the next 2-3 h the rate of alkali addition decreased and practically ceased. The culture medium, which was turbid, was sampled at this time and about 200 mg wet weight of sediment was deposited by centrifugation of 30 ml of the culture medium. This sediment was creamy in appearance with no semblance of structural organisation.

Minutes after sucrose was added to the culture, there was a sharp increase in the rate of alkali intake to the culture, demonstrating an increase in metabolic activity. The culture medium became more turbid and there was an increased yield of sediment from the samples removed 1.25 and 2.5 h after sucrose addition (300-400 mg wet weight of sediment from 30 ml of culture medium). These sediments were firmer than the glucose-grown samples and there was aggregation of bacteria. Eventually, the bacterial aggregates formed larger clumps within the culture. This clumping

began for some cultures within 6 h of sucrose addition, but for others appeared overnight between 6 and 23 h after sucrose addition. There was a greatly increased yield of sediment from these samples (1-2 g wet weight from 30 ml of culture medium).

The water content of the sediments was found by drying to range from 82.3 to 93.1 per cent by weight, with a mean value of 88.4 per cent.

7.3.2 Diffusion of NaF in Bacterial and Glucan Sediments

Table 7.1 lists diffusion coefficients of NaF measured at 37°C in bacterial sediments from seven cultures of S. sanguis 804. Also listed are the measurements of the total carbohydrate and DNA contents of these sediments (expressed as µg/mg dry weight of sediment) and the calculated carbohydrate to DNA ratios. The DNA assays failed for cultures 3 and 4 and the carbohydrate assay failed for culture 4. As each culture progressed, the total carbohydrate concentration and the carbohydrate to DNA ratio increased, whilst the DNA concentration, which indicates the proportion of bacterial cells in the sample, decreased.

It was primarily intended to compare diffusion coefficients to the carbohydrate contents and the carbohydrate to DNA ratios of the sediments. However, it was observed that as the sucrose-containing cultures progressed, the sediments became gelatinous in appearance, and these sediments have been marked with an asterisk in Table 7.1. As it has been suggested (see Section 2.3) that rates of diffusion are restricted in gelatinous plaques, diffusion

Table 7.1 Diffusion coefficients of NaF in sediments of S. sanguis 804 at 37°C, and the total carbohydrate and DNA concentrations in the sediments.

Sample	Total carbohydrate (µg/mg dry wt.)	Total DNA (µg/mg dry wt.)	Ratio of carbohydrate to DNA	Diffusion coefficient (cm ² s ⁻¹) x 10 ⁶
<u>Culture No. 1</u>				
16 h glucose	110	67	1.6	4.52
1.25 h sucrose	193	57	3.4	4.92
2.5 h sucrose	262	51	5.1	4.20
6 h sucrose*	535	51	10.5	15.4
<u>Culture No. 2</u>				
16 h glucose	205	87	2.4	4.92
1.25 h sucrose	246	67	3.7	4.75
2.5 h sucrose	514	53	9.7	7.53
6 h sucrose*	583	30	19.4	10.3
<u>Culture No. 3</u>				
16 h glucose	133	-	-	6.00
1.25 h sucrose	251	-	-	5.54
2.5 h sucrose	239	-	-	5.83
6 h sucrose	472	-	-	4.82
23 h sucrose*	507	-	-	10.7

(Cont.)

Table 7.1 (Cont.)

Sample	Total carbohydrate ($\mu\text{g}/\text{mg}$ dry wt.)	Total DNA ($\mu\text{g}/\text{mg}$ dry wt.)	Ratio of carbohydrate to DNA	Diffusion coefficient (cm^2s^{-1}) $\times 10^6$
<u>Culture No. 4</u>				
16 h glucose	-	-	-	5.31
1.25 h sucrose	-	-	-	4.85
2.5 h sucrose	-	-	-	6.37
6 h sucrose	-	-	-	4.76
24 h sucrose*	-	-	-	13.9
<u>Culture No. 5</u>				
16 h glucose	113	50	2.3	4.74
1.25 h sucrose	130	36	3.6	4.85
19 h sucrose*	527	46	11.5	12.3
<u>Culture No. 6</u>				
16 h glucose	131	78	1.7	4.96
1.25 h sucrose	191	60	3.2	4.86
2.5 h sucrose	230	59	3.9	4.29
6 h sucrose*	543	43	12.6	7.53
<u>Culture No. 7</u>				
16 h glucose	64	54	1.2	4.58
3.75 h sucrose	178	27	6.6	5.79
4.75 h sucrose*	267	16	16.7	7.86
6 h sucrose*	373	13	28.7	7.02

*indicates that sample was gelatinous in appearance (see text)

coefficients in gelatinous and non-gelatinous sediments will be compared.

The clearance curves from the diffusion experiments were single exponential and the correlation coefficients from the regression analyses were all greater than 0.98 ($p < 0.001$).

NaF diffusion coefficients are plotted against the total carbohydrate concentrations in the sediments in Fig. 7.2. Linear regression analysis revealed a significant positive correlation between diffusion coefficient and total carbohydrate concentration ($r = 0.753$, $p < 0.001$), although this does not prove that the relationship is linear. The sediments were divided into a high and a low carbohydrate-containing group. The low carbohydrate group ($< 300 \mu\text{g}/\text{mg}$ dry weight), which had about the same range of carbohydrate concentrations as reported for plaque (details to be given in Section 7.4), gave a mean value of NaF diffusion coefficient, \pm standard deviation, of $(5.16 \pm 0.90) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. Corresponding values for the high carbohydrate-containing group ($> 350 \mu\text{g}/\text{mg}$ dry weight) were $(9.45 \pm 3.39) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. When compared using Student's t-test, these two groups were found to have significantly different values of diffusion coefficient ($t = 3.533$; $0.005 > p > 0.001$; Table 7.2), diffusion being almost twice as fast in the high carbohydrate group.

All but two of the high carbohydrate-containing sediments were very gelatinous in appearance. Using all the available data from Table 7.1, the mean diffusion coefficient of NaF, \pm standard deviation, for gelatinous-appearing sediments was

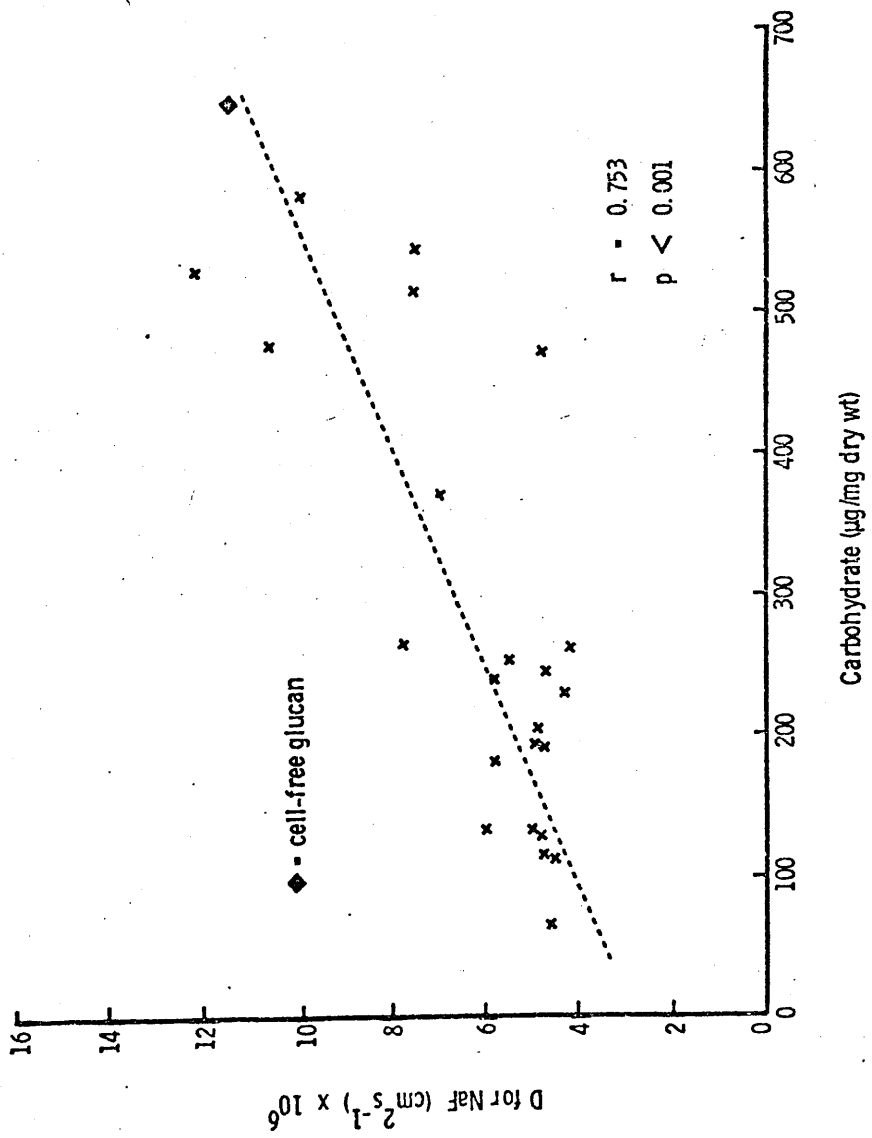


Fig. 7.2 Diffusion coefficient of NaF in sediments of Streptococcus sanguis as a function of the total carbohydrate concentration in the sediments.

Table 7.2 Statistical comparisons of diffusion coefficients of NaF in various groups of bacterial sediment using Student's t-test.

Type of sediment	Diffusion coefficient (cm^2s^{-1}) $\times 10^6$ mean \pm S.D.	n	t	p
low carbohydrate (< 300 $\mu\text{g}/\text{mg}$ dry wt.)	5.16 \pm 0.90	16	3.533	0.005 > p > 0.001
high carbohydrate (> 350 $\mu\text{g}/\text{mg}$ dry wt.)	9.45 \pm 3.39	8		
non-gelatinous in appearance	5.16 \pm 0.78	21	4.947	p < 0.001
gelatinous in appearance	10.63 \pm 3.09	8		

n = number of measurements

t = Student's t-parameter

p = probability level

$(10.63 \pm 3.09) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and for non-gelatinous sediments was $(5.16 \pm 0.78) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. Student's t-test reveals that these values are significantly different ($t = 4.947$; $p < 0.001$; Table 7.2), again by a factor of about two.

When linear regression analysis was applied to the low carbohydrate samples, no significant correlation was found between NaF diffusion coefficient and carbohydrate concentration ($r = 0.310$, $p > 0.2$).

A similar pattern of results was obtained when the diffusion coefficient was plotted against the ratio of carbohydrate to DNA in the sample (Fig. 7.3). High values of diffusion coefficient were found in samples with carbohydrate to DNA ratios greater than eight. Linear regression analysis revealed a positive correlation between NaF diffusion coefficient and carbohydrate to DNA ratio ($r = 0.533$, $0.025 > p > 0.001$).

The measured values of total carbohydrate, DNA and NaF diffusion coefficients in bacterial-free glucan material are listed in Table 7.3. Carbohydrate concentrations were in excess of 500 $\mu\text{g}/\text{mg}$ dry weight and the mean value of diffusion coefficient, \pm standard deviation, was $(11.55 \pm 1.39) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. This value of diffusion coefficient shows no statistically significant difference, when compared using Student's t-test, with the values found for the high carbohydrate group ($t = 1.516$, $p > 0.1$) and for the gelatinous group ($t = 0.711$, $p > 0.4$) of sediments.

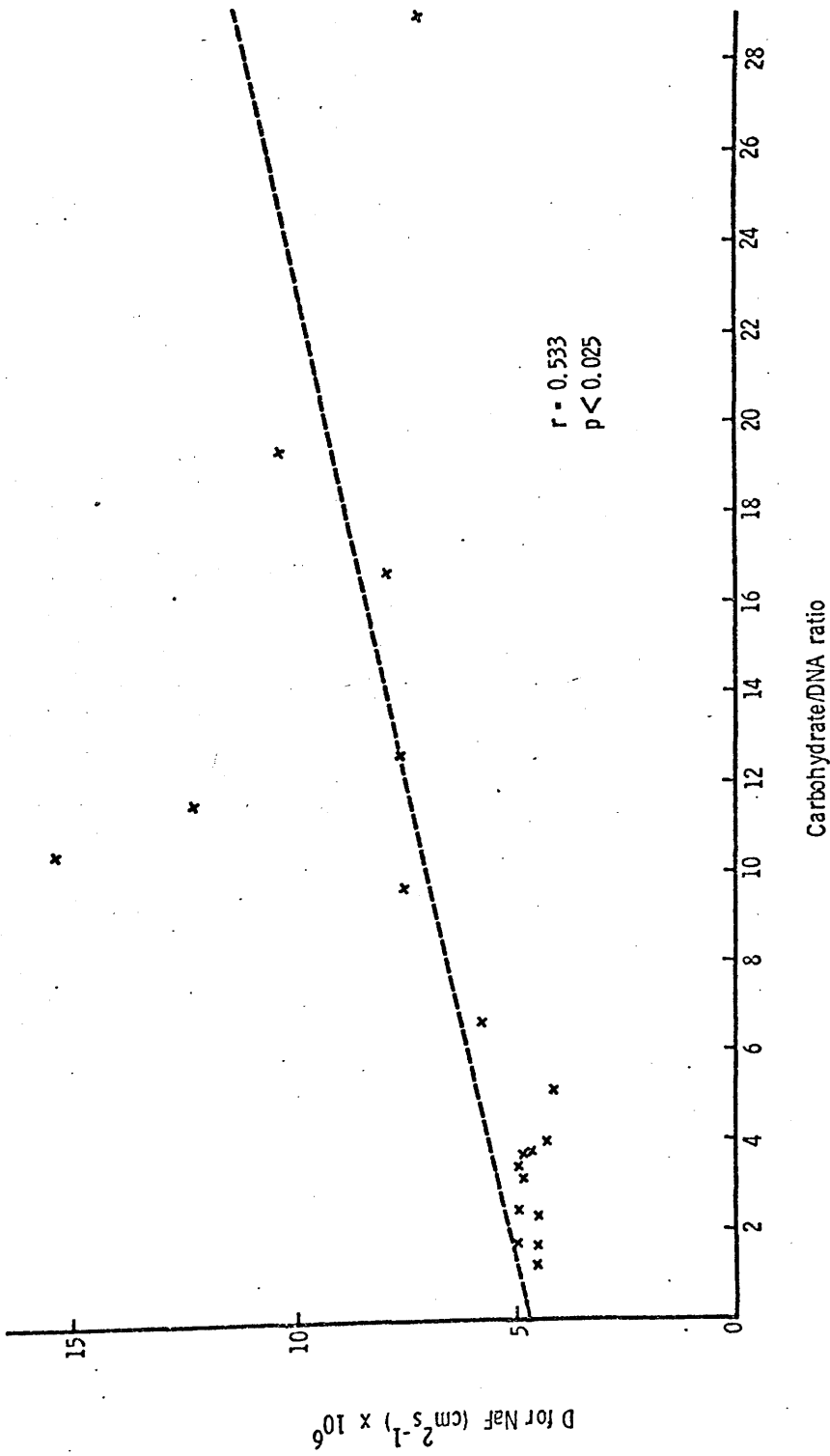


Fig. 7.3 Diffusion coefficient of NaF in sediments of *Streptococcus sanguis* as a function of the ratio of total carbohydrate to DNA concentration in the sediments.

Table 7.3 Diffusion coefficients of NaF in bacteria-free glucan material at 37°C, and the total carbohydrate and DNA concentrations in the samples.

Culture Number	Total carbohydrate (µg/mg dry wt.)	Total DNA (µg/mg dry wt.)	Ratio of carbohydrate to DNA	Diffusion coefficient (cm ² s ⁻¹) x 10 ⁶
7	543	3.7	147	12.2
	568	6.6	86.1	10.3
	830	11.1	74.8	*13.2 10.5

*two measurements

Mean diffusion coefficient ± standard deviation = (11.55 ± 1.39) x 10⁻⁶ cm² s⁻¹

7.3.3 Diffusion of ^{14}C -Sucrose and NaF in Glutaraldehyde-Fixed Bacterial Sediments

Table 7.4 lists the diffusion coefficients measured at 37°C for ^{14}C -sucrose and NaF in glutaraldehyde-fixed bacterial sediments from three different cultures of S. sanguis 804. Also listed are measurements of the total carbohydrate and DNA contents of the sediments (expressed as $\mu\text{g}/\text{mg}$ dry weight) and the calculated ratios of total carbohydrate to DNA. As each culture progressed, the carbohydrate concentration and the carbohydrate to DNA ratio for the samples increased, and the DNA concentration decreased. Gelatinous-appearing samples are marked in Table 7.4 with an asterisk. The clearance curves from the diffusion experiments were single exponential and the correlation coefficients from the regression analyses were all greater than 0.93 ($p < 0.001$) for sucrose experiments, and greater than 0.98 ($p < 0.001$) for NaF experiments.

Fig. 7.4 shows a plot of the diffusion coefficients for ^{14}C -sucrose and NaF against the total carbohydrate concentrations in the samples. Linear regression analysis again revealed a significant positive correlation between the NaF diffusion coefficient and the carbohydrate concentration ($r = 0.780$, $0.005 > p > 0.001$), but correlation between the ^{14}C -sucrose diffusion coefficient and the carbohydrate concentration just failed to reach statistical significance ($r = 0.551$, $0.1 > p > 0.05$).

The mean value, \pm standard deviation, of the NaF diffusion coefficient for 10 samples with low carbohydrate concentrations ($< 200 \mu\text{g}/\text{mg}$ dry weight) was $(4.29 \pm 0.55) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. This

Table 7.4 Diffusion coefficients, D, of NaF and ^{14}C -sucrose in glutaraldehyde-fixed sediments from *S. sanguis* 804 cultures, measured at 37°C. Also, the total carbohydrate and DNA concentrations in the sediments.

Sample	Total carbohydrate ($\mu\text{g}/\text{mg}$ dry wt.)	Total DNA ($\mu\text{g}/\text{mg}$ dry wt.)	Ratio of carbohydrate to DNA	D for NaF (cm^2s^{-1}) $\times 10^6$	D for ^{14}C -sucrose (cm^2s^{-1}) $\times 10^6$
<u>Culture No. 8</u>					
16 h glucose	79	50	1.6	5.49	3.84
1.25 h sucrose	119	34	4.0	4.51	2.61
2.5 h sucrose	114	28	4.1	4.03	2.63
6 h sucrose*	348	18	19.0	8.60	4.01
<u>Culture No. 9</u>					
16 h glucose	82	53	1.5	4.21	2.85
1.25 h sucrose	132	38	3.5	4.11	3.09
2.5 h sucrose	169	40	4.2	4.11	2.24
6 h sucrose*	418	23	18.2	7.50	-
<u>Culture No. 10</u>					
16 h glucose	82	60	1.4	4.02	3.23
1.25 h sucrose	87	48	1.8	4.44	3.57
2.5 h sucrose	112	44	2.5	4.58	2.92
6 h sucrose	145	39	3.7	3.35	2.82
23 h sucrose*	423	7.1	59.6	5.93	4.13

*indicates that sample was gelatinous in appearance (see text)

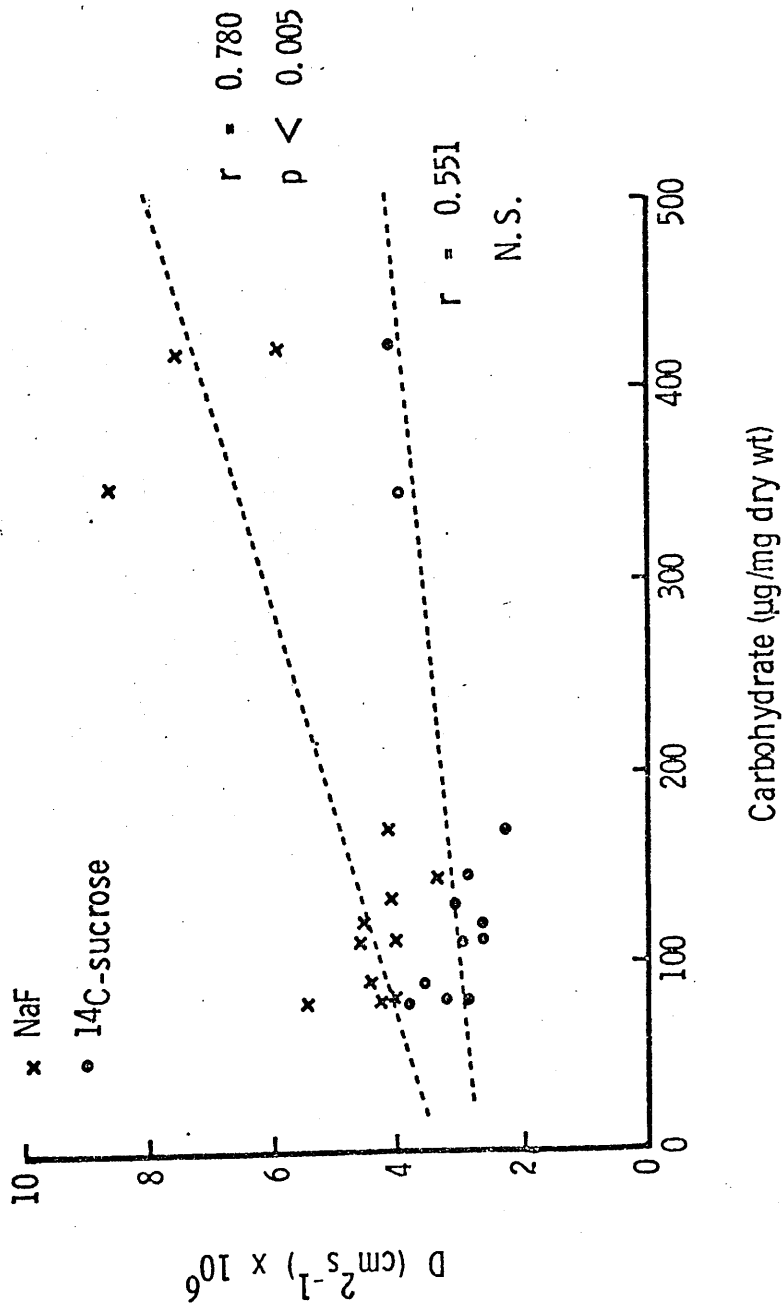


Fig. 7.4 Diffusion coefficient of NaF and ¹⁴C-sucrose in glutaraldehyde-fixed sediments of Streptococcus sanguis as a function of the total carbohydrate concentration in the sediments.

value is about 17 per cent lower than the value reported in Section 7.3.2 for diffusion of NaF in non-fixed, low carbohydrate samples, and this difference is statistically significant when tested using Student's t-test ($t = 3.059$, $p < 0.01$; Table 7.5).

The mean value, \pm standard deviation, of diffusion coefficient for ^{14}C -sucrose in low carbohydrate samples ($< 200 \mu\text{g}/\text{mg}$ dry weight) was $(2.98 \pm 0.47) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$.

^{14}C -sucrose diffusion coefficients were measured for only two gelatinous samples, each with more than $300 \mu\text{g}$ carbohydrate/mg dry weight. These diffusion coefficients were 4.01×10^{-6} and $4.13 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, which are about 35 per cent higher than the values for the low carbohydrate group.

7.4 DISCUSSION

The rate of growth of Streptococcus sanguis 804 in the batch cultures was not precisely reproducible. This may have been due to non-standardisation of the starter culture, and might have been reduced by monitoring the turbidity of the inoculum. Consequently, rather than comparing samples according to the time at which they were harvested, more emphasis should be placed upon their physical appearance and chemical and biochemical composition.

The rate of diffusion of NaF was positively correlated with the sample total carbohydrate content, much of which is expected to be glucan (Beeley and Black, 1977). Diffusion in sediments of high total carbohydrate content was about twice as fast as in sediments of low total carbohydrate, for which the NaF diffusion coefficient was

Table 7.5 Statistical comparison of diffusion coefficients of NaF and ^{14}C -sucrose in plaque and in sediments from S. sanguis 804 cultures, using Student's t-test

Sample	Diffusing substance	Diffusion coefficient (cm^2s^{-1}) $\times 10^6$ mean \pm S.D.	n	t	p
plaque	NaF	$4.24 \pm 0.50^+$	22	-3.695	$p < 0.001$
<u>S. sanguis</u> sediment, low carbohydrate	NaF	5.16 ± 0.90	16		
<u>S. sanguis</u> sediment, low carbohydrate, glutaraldehyde-fixed	NaF	4.29 ± 0.55	10	-3.059	$0.01 > p > 0.005$
plaque, glutaraldehyde-fixed	^{14}C -sucrose	$3.0 \pm 0.3^{++}$	4	-0.095	$p > 0.5$
<u>S. sanguis</u> sediment, low carbohydrate, glutaraldehyde-fixed	^{14}C -sucrose	2.98 ± 0.47	10		

n = number of measurements

t = Student's t-parameter

p = probability level

+ = Chapter 4

++ = Chapter 5

$5.16 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. This diffusion coefficient is only about 20 per cent higher than the value reported in Chapter 4 for diffusion of NaF in plaque ($4.24 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$), although this difference is statistically significant when tested by Student's t-test ($t = 3.695$, $p < 0.001$; Table 7.5). These sediments, with less than 300 μg total carbohydrate/mg dry weight, bear some resemblance to plaque. The mean water content of the sediments was 88.4 per cent by weight, which is only a little greater than the value of 82 per cent reported for plaque by Edgar and Tatevossian (1971). The total carbohydrate content of plaque appears to vary according to the plaque age, its exposure to sucrose and the time of sampling. Wood (1967) measured total soluble polysaccharide levels of 60 $\mu\text{g}/\text{mg}$ dry weight for 24 h accumulations of plaque. Hotz, Guggenheim and Schmid (1972), using plaque pooled from schoolchildren, measured 182 μg total carbohydrate/mg dry weight. Ashley and Wilson (1977) reported total carbohydrate concentrations of 117 $\mu\text{g}/\text{mg}$ dry weight in plaque accumulated for 48 h by subjects on sugar-free diets and 321 $\mu\text{g}/\text{mg}$ dry weight in mature plaque of unknown age. Holm-Pedersen, Folke and Gawronski (1980) measured 338 and 249 μg total carbohydrate/mg dry weight for 4-day and 9-day accumulations of plaque respectively, for young adult subjects (20-24 years of age). Corresponding results for plaque from elderly subjects (65-81 years of age) were 248 and 434 μg carbohydrate/mg dry weight. This range of carbohydrate concentrations incorporates the values measured for the low carbohydrate group of sediments.

The rate of diffusion of NaF in the bacteria-free glucan material was the same as in the high carbohydrate group of sediments. It would appear that rates of diffusion in these sediments are

regulated by the glucan rather than by the cells. This may happen to some extent in plaque although these sediments contain higher concentrations of carbohydrate than have been reported for plaque.

Glutaraldehyde-fixation of the low carbohydrate-containing sediments resulted in a small (17 per cent) but statistically significant reduction in the rate of diffusion of NaF (Table 7.5). This is in contrast to the results reported in Chapter 5, where glutaraldehyde-fixation of plaque led to a slight, but statistically insignificant, increase in the rate of diffusion of NaF. However, using the Mann-Whitney U-test (Siegel, 1956), the total carbohydrate concentrations of the sediments used in the glutaraldehyde-fixation experiments were found to be significantly lower than for the non-fixed sediments ($U = 29, p < 0.01$). Therefore, because the NaF diffusion coefficient is correlated with the carbohydrate concentration, it is not possible to deduce the precise effect of sample fixation upon the rate of diffusion of NaF, but merely to observe that the effect is small. This is also true for sediments of high total carbohydrate concentrations.

The diffusion coefficient of ^{14}C -sucrose in the low carbohydrate group of sediments ($2.98 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) is not significantly different from the diffusion coefficient of ^{14}C -sucrose in plaque, reported in Chapter 5 to be $3.0 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Table 7.5). Compared to NaF, diffusion of sucrose in bacterial sediments was relatively independent of carbohydrate concentration. However, sucrose diffusion coefficients were measured for only 12 sediments and for the two gelatinous sediments diffusion coefficients were about 35 per cent greater than for the low carbohydrate sediments.

Another point to consider is that the diffusion coefficient of NaF in water at 37°C is $18.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, but for sucrose is $7.0 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (see Appendix). Therefore the ratio of diffusion coefficient in water compared to the diffusion coefficient in the low carbohydrate sediments is 3.6 for NaF but only 2.3 for sucrose. The effect of glucan on diffusion of sucrose may be proportionally smaller than for NaF, and therefore less easily detectable.

Tatevossian (1979) measured the diffusion coefficient of sucrose in plaque sediment, which he separated from plaque by centrifuging at 5000 g for 15 min, the same treatment as used for the S. sanguis sediments. Tatevossian reported a diffusion coefficient of $0.92 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, which is slower by a factor of 3.2 than the values reported in this thesis for S. sanguis sediments and for plaque. This discrepancy can be accounted for by examining the technique used by Tatevossian. He allowed ^{14}C -sucrose to penetrate into a column of plaque sediment, and from penetration profiles calculated an effective diffusion coefficient. However, as the sucrose penetrates into the sediment, some of it may be metabolised and some will bind to the constituents of the sediment, such as cell walls and matrix. Thus the process Tatevossian studied was not one of pure diffusion. Many of these detrimental effects can be circumvented by monitoring clearance of sucrose from the sample, as reported in this thesis.

To summarise, the results reported in this chapter are the first direct measurements of the effect of glucan on rates of diffusion. It has been shown that glucan does not act as a barrier to diffusion of NaF or sucrose. Diffusion coefficients of NaF and ^{14}C -sucrose varied only slightly in sediments of low total

carbohydrate concentration ($< 300 \mu\text{g}/\text{mg}$ dry weight). The NaF diffusion coefficient was positively correlated with the sample carbohydrate content. Diffusion of NaF in gelatinous-appearing sediments of high carbohydrate content and in bacteria-free glucan material was twice as fast as in low carbohydrate sediments or in plaque. These results are in contrast to some commonly held, and widely published points of view.

CHAPTER 8

RATE OF DIFFUSION OF NaF IN DENTAL PLAQUE IN RELATION TO PLAQUE CARBOHYDRATE CONTENT

8.1 INTRODUCTION

It was shown in Chapter 7 that in sediments from cultures of Streptococcus sanguis 804 (NCTC 10904), the rate of diffusion of NaF increased as the glucan content of the sediments increased. There was also a tendency for sucrose to diffuse faster in sediments with high glucan content. There are, as yet, no quantitative measurements of the effect of extracellular polysaccharides on rates of diffusion in plaque, despite many claims that extracellular polysaccharides form a diffusion barrier within plaque (see Section 2.3).

The experimental techniques reported in this thesis are suitable for measuring diffusion coefficients in the small amounts of plaque accumulated in the mouths of individual subjects. Therefore, the aims of the experiments reported in this chapter were two-fold. The first experiment was designed to relate the diffusion coefficient of NaF in plaque from individual subjects to the total carbohydrate concentration in the plaque samples. To ensure a wide range of plaque carbohydrate concentration, measurements were made on plaque of unknown age collected from patients attending the Glasgow Dental Hospital and School, as well as plaque of 24 h accumulation. In the second experiment, plaque samples from three subjects were incubated in sucrose-containing broth in vitro. This should result

in the production of large amounts of polysaccharide in the plaque samples. Diffusion coefficients were measured for NaF in these plaque samples before and after incubation.

8.2 METHODS

8.2.1 Measurement of the NaF Diffusion Coefficient in Plaque in Relation to Plaque Carbohydrate Content.

Plaque for use in this experiment was obtained from two sources. Five young adult members of staff of the Glasgow Dental Hospital and School (subjects SM, HMA, DW, DE and LS) with controlled, moderate rates of caries, each accumulated plaque by refraining from tooth cleaning for 24 h and, as in previous experiments, plaque was collected at least 1.5 h after the subject's last consumption of food or drink. Plaque of indeterminate age was collected from ten patients attending the Glasgow Dental Hospital and School. Four of these patients were adults, aged between 26 and 52 years (subjects A, B, C and G) and the other six were children aged from 8.5 to 14 years (subjects D, E, F, H, I and J). The patients were suffering from some degree of gingivitis and dental caries; plaque was scraped from all available sites excluding areas of calculus, using a nickel micro-spatula. Each of the subjects (patients and staff) yielded at least 20 mg of plaque wet weight, suggesting that the standard of oral hygiene of the patients was poor. Each of the children admitted to eating moderate or high quantities of sweets.

On collection, plaque samples were immediately stored on

ice but were used individually for diffusion experiments within 30 min. Approximately 12 mg of the plaque were placed in the plaque assembly described in Section 4.2.2 and equilibrated for 12 min at 37°C in 10 ml of 7 mM K_2HPO_4 - KH_2PO_4 buffer (pH 6.8) with 2.2 per cent NaF (resultant pH = 6.6). The diffusion coefficient of NaF in the plaque samples was determined by monitoring the clearance of fluoride from the plaque into 10 ml of NaF-free buffer, as described in detail in Section 4.2. The remainder of the plaque (in excess of 5 mg wet weight) was retained for assay of total carbohydrate using the cysteine-sulphuric acid colorimetric reaction described in Section 7.2.2, and for DNA (which reflects the cellular content of the plaque) using the diphenylamine colorimetric reaction described in Section 7.2.3.

8.2.2 Measurement of the NaF Diffusion Coefficient in Plaque Incubated in Sucrose-Containing Broth in vitro.

For this set of experiments, plaque was collected from three of the subjects (SM, HMA and LS) who each accumulated about 30 mg of plaque wet weight by refraining from tooth cleaning for 24 h. Plaque was collected as described in Section 8.2.1 and tested individually. About 5 mg of plaque were retained for assay of total carbohydrate and DNA and about 12 mg of plaque were placed in each of two brass dishes as described in Section 4.2.2. To hold the plaque in the dish during incubation and to keep the volume of the plaque constant, a stainless-steel support screen from a Millipore filter-holder (Millipore U.K. Ltd., Wembley, Middlesex, England; Catalogue number XX30 012 03) was retained over a Millipore filter and the plaque using a wire frame. Apart from the inclusion of the support screen, the plaque assembly was as

described in Section 4.2.2. The support screen, 0.17 mm thick, was rigid and prevented bulging of the filter. The pores in the support screen were about 0.2 mm in diameter and accounted for about 50 per cent of the surface area of the screen.

The two plaque assemblies were suspended in 100 ml of Brain Heart Infusion Broth (Gibco Bio-Cult Diagnostics Ltd., Paisley, Scotland) with 5 per cent (w/v) sucrose. The broth was continuously stirred using a magnetic stirrer and incubated aerobically at 37°C for 16 h. The pH of the broth was measured before and after incubation. Plaque was removed from one of the plaque assemblies after incubation and assayed for total carbohydrate and DNA. The other plaque assembly was used, with the support screen still in position, for a diffusion experiment. The assembly was suspended for 20 min at 37°C in 10 ml of the 7 mM potassium phosphate buffer with 2.2 per cent NaF. Clearance of fluoride from the plaque sample into 10 ml of the NaF-free buffer was then monitored for a period of 12 min (0.1 ml aliquots being removed after 2,3,4,5,6,8,10 and 12 min) and the NaF diffusion coefficient was calculated as described in Section 4.2.

To test whether the presence of the support screen affected the measured value of diffusion coefficient, a control experiment was performed in which the NaF diffusion coefficient was measured as described above, using plaque collected from each subject on a separate occasion and which was not incubated in sucrose-containing broth.

8.3 RESULTS

8.3.1 Diffusion Coefficient of NaF in Relation to Plaque Carbohydrate Content.

Table 8.1 lists the measured values of the NaF diffusion coefficient in the plaque from each subject, together with the total carbohydrate and DNA concentrations and the ratio of total carbohydrate to DNA. The mean NaF diffusion coefficient, \pm standard deviation, was $(4.32 \pm 0.53) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ which is not significantly different from the value of $(4.24 \pm 0.50) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ reported in Chapter 4 for the diffusion coefficient of NaF in plaque (Student's t-test, $t = 0.461$, $p > 0.5$).

The concentration of total carbohydrate in the plaques ranged from 62 to 451 μg carbohydrate/mg plaque dry weight, with a mean value of 158 $\mu\text{g}/\text{mg}$. Patients, particularly the children, tended to have higher plaque carbohydrate levels than the staff members. The NaF diffusion coefficient is plotted against the plaque total carbohydrate concentration in Fig. 8.1. Linear regression analysis revealed a statistically significant positive correlation ($r = 0.560$, $t = 2.440$, $0.05 > p > 0.025$) i.e. the rate of diffusion increased as the carbohydrate concentration increased. The plaque from one of the patients had a much higher carbohydrate content (451 $\mu\text{g}/\text{mg}$ dry weight) relative to the other samples. Omitting the data from this subject from the regression analysis, the correlation fails to reach statistical significance ($r = 0.328$, $t = 1.201$, $p > 0.2$) although the values for the intercept and gradient of the best-fitting straight line changed only slightly.

Table 8.1 Diffusion coefficients of NaF in dental plaque at 37°C and the plaque total carbohydrate, DNA and carbohydrate to DNA ratio.

Subject	Total carbohydrate ($\mu\text{g}/\text{mg}$ dry wt.)	DNA ($\mu\text{g}/\text{mg}$ dry wt.)	Ratio of carbohydrate to DNA	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$
HMA	62	40	1.6	3.89
SM	76	43	1.8	4.30
DE	90	41	2.2	4.43
DW	134	45	3.0	3.83
LS	180	38	4.7	4.75
A	100	39	2.6	4.09
B	112	42	2.7	4.81
C	116	28	4.1	3.11
D	133	62	2.1	4.78
E	143	54	2.6	4.16
F	148	43	3.4	4.12
G	183	73	2.5	3.96
H	215	45	4.8	4.84
I	230	42	5.5	4.47
J	451	53	8.5	5.24
mean	158	46	3.5	4.32
S.D.	94	11	1.8	0.53

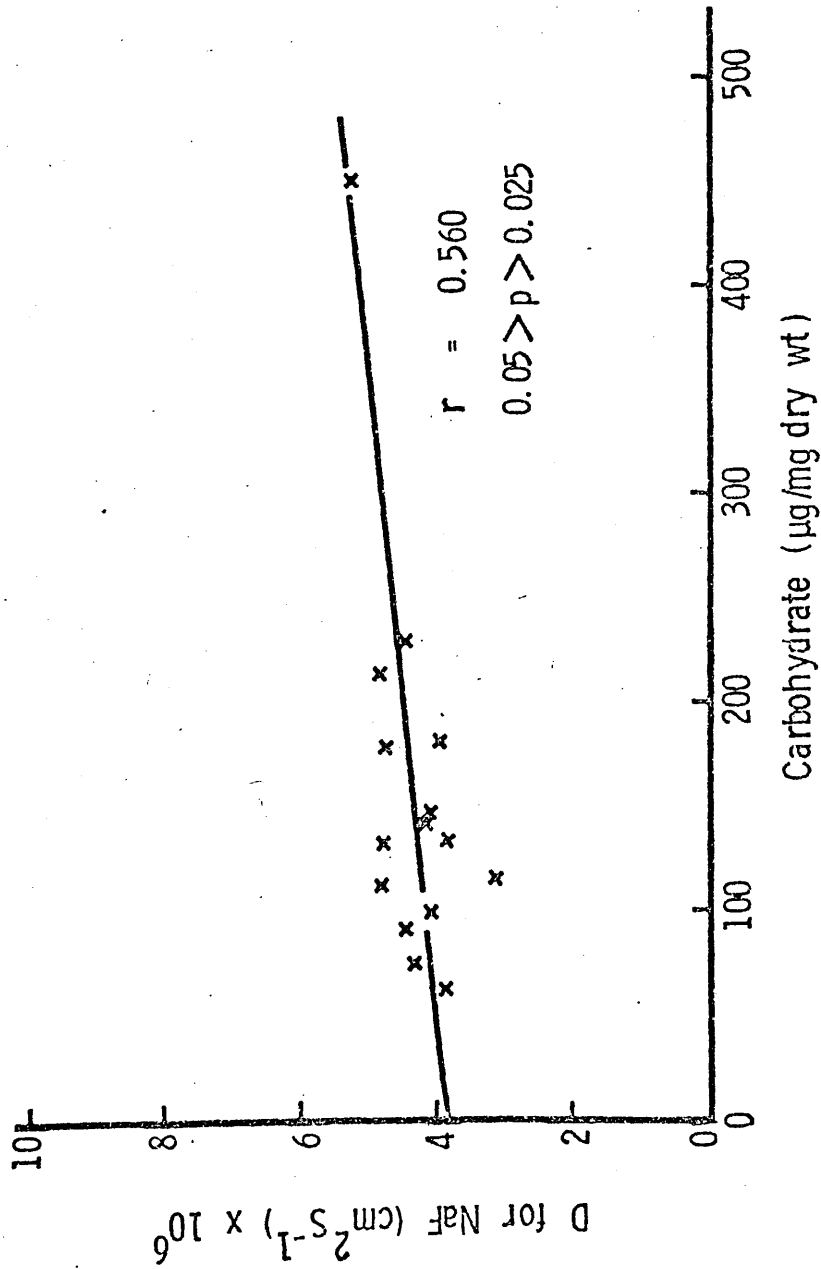


Fig. 8.1.1 Diffusion coefficient of NaF in plaque as a function of the total carbohydrate concentration in the plaque.

The plaque DNA concentrations varied less than the carbohydrate concentrations, ranging from 28 to 73 $\mu\text{g DNA/mg dry weight}$ with a mean value, \pm standard deviation, of $46 \pm 11 \mu\text{g/mg}$. Linear regression analysis showed no significant correlation between the plaque DNA and carbohydrate concentrations ($r = 0.296$, $t = 1.117$, $p > 0.2$) but a highly significant positive correlation between the carbohydrate to DNA ratio and the carbohydrate concentration ($r = 0.925$, $t = 8.777$, $p < 0.001$). There was no significant correlation between the NaF diffusion coefficient and the carbohydrate to DNA ratio ($r = 0.447$, $t = 1.802$, $0.10 > p > 0.05$).

8.3.2 Diffusion Coefficient of NaF in Plaque Incubated in Sucrose-Containing Broth.

During incubation, the pH of each of the broths dropped from 7.40 ± 0.05 to 6.85 ± 0.05 . The measured values of NaF diffusion coefficient, the plaque total carbohydrate and DNA concentration and the ratio of carbohydrate to DNA are listed in Table 8.2. The samples labelled 'control' refer to the experiments performed without incubation of the plaque. 'Pre-sucrose' and 'post-sucrose' refer to measurements made on plaque samples before and after incubation in sucrose-containing broth respectively.

The mean value of the NaF diffusion coefficient for the control experiment, designed to test the effect of the presence of the support screen, was $3.29 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. This is less than the value of $4.24 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ reported in Chapter 4 for the diffusion coefficient of NaF in plaque. For two of the subjects (SM and LS) the diffusion coefficients reported for the control experiment in

Table 8.2 The effect of incubation of plaque in sucrose-containing broth on the NaF diffusion coefficient and on the plaque total carbohydrate and DNA concentrations.

'Control' measurements were made without incubation of plaque. 'Pre-sucrose' and 'post-sucrose' measurements were made on plaque samples before and after incubation in sucrose-containing broth respectively (see text for full details).

Subject	Sample	Total carbohydrate ($\mu\text{g}/\text{mg}$ dry wt.)	DNA ($\mu\text{g}/\text{mg}$ dry wt.)	Ratio of carbohydrate to DNA	Diffusion coefficient (cm^2s^{-1}) x 106
SM	control	61	35	1.7	2.82
	pre-sucrose	82	44	1.9	-
	post-sucrose	249	55	4.5	4.01
HMA	control	210	56	3.7	3.97
	pre-sucrose	70	46	1.5	-
	post-sucrose	360	77	4.7	4.30
LS	control	194	38	5.1	3.08
	pre-sucrose	162	72	2.2	-
	post-sucrose	463	86	5.4	3.61

Table 8.2, are smaller than the values reported for the same subjects in Table 8.1, although the plaque carbohydrate concentrations are similar in each case. For the third subject (HMA) the diffusion coefficient reported in Table 8.2 for the control experiment is slightly larger than the corresponding value listed in Table 8.1, but in this case the plaque carbohydrate concentration is much higher in the control test (210 $\mu\text{g}/\text{mg}$ dry weight versus 62 $\mu\text{g}/\text{mg}$ dry weight). These results suggest that the presence of the support screen in the plaque assembly leads to a small reduction in the measured value of NaF diffusion coefficient, although this effect may be masked by variations in plaque carbohydrate concentration.

The mean NaF diffusion coefficient for samples incubated in sucrose-containing broth was $3.97 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. For each of the three subjects diffusion was faster in these post-sucrose samples than in the control tests. Comparison of the results for pre-sucrose and post-sucrose samples in Table 8.2 reveals an increase of between three- and five-fold in the plaque total carbohydrate concentration during incubation. This was accompanied by an increase of between 20 and 65 per cent in the DNA concentration and a two- to three-fold increase in the ratio of carbohydrate to DNA. The carbohydrate and DNA concentrations are similar for the control and pre-sucrose samples: the only large difference was for subject HMA where the total carbohydrate concentration was 210 $\mu\text{g}/\text{mg}$ dry weight for the control sample and 70 $\mu\text{g}/\text{mg}$ dry weight for the pre-sucrose sample.

8.4 DISCUSSION

The range of plaque carbohydrate concentration listed in Tables 8.1 and 8.2 is in agreement with values reported by Wood (1967), Hotz, Guggenheim and Schmid (1972), Ashley and Wilson (1977) and Holm-Pedersen, Folke and Gawronski (1980). As was summarised in Section 7.4, these workers measured plaque carbohydrate concentrations ranging from 60 to 434 $\mu\text{g}/\text{mg}$ dry weight. The mean plaque DNA concentration from Table 8.1 (46 $\mu\text{g}/\text{mg}$ dry weight) is slightly higher than the value of 26.5 $\mu\text{g}/\text{mg}$ dry weight measured by Drummond and Donkersloot (1974) for four-day accumulations of plaque from subjects on sucrose-supplemented diets, for whom the plaque polysaccharide concentrations ranged from 81 to 142 $\mu\text{g}/\text{mg}$ dry weight. The carbohydrate concentration in one of the plaque samples reported in Table 8.1 was 451 $\mu\text{g}/\text{mg}$ dry weight which is considerably greater than for the other samples. This may have been due to the presence of food debris in the plaque, but these can normally be identified visually and it may be that the plaque was exposed to carbohydrate shortly before collection, resulting in high concentrations of stored intra- and extra-cellular polysaccharides. The measurements reported in this chapter show that the plaque DNA concentration, which gives an indication of the cellular content of the plaque, is less variable than the carbohydrate concentration.

The aim of the experiment described in Section 8.2.1 was to investigate the relationship between the rate of diffusion of NaF in dental plaque and the carbohydrate content of the plaque. The results, based on measurements using 15 plaque samples

representative of the known range of plaque carbohydrate concentration, indicate a low correlation between the NaF diffusion coefficient and the carbohydrate concentration, but with a tendency for slightly faster diffusion with increasing carbohydrate concentration. This is in agreement with the results reported in Chapter 7 for diffusion of NaF in sediments of Streptococcus sanguis with carbohydrate concentrations of less than 300 µg/mg dry weight, although diffusion was about 20 per cent faster in the sediments than in plaque.

The results of the experiments described in Section 8.2.2 show that the presence of the support screen leads to a slight reduction in the calculated value of the NaF diffusion coefficient. This may be explained by the trapping of a stagnant layer of fluid within the pores of the support screen, leading to an increase in the effective thickness of the sample. The decrease in the pH of the broth and the increases in the plaque carbohydrate and DNA concentrations show that the plaque bacteria were metabolically active during the incubation. The large increase in plaque carbohydrate concentration is likely to be due to synthesis of intra- and extra-cellular polysaccharides by the plaque bacteria, although there may be small amounts of sucrose, glucose and fructose remaining. The increase in plaque DNA indicates cell division during incubation, and because the volume of the plaque was constant, this suggests an increase in the packing density of bacteria in the plaque. Despite this increased packing density, there was a small increase in the rate of diffusion of NaF in each of the three plaques after incubation. This is consistent with the increase of

NaF diffusion coefficient with increasing plaque carbohydrate concentration reported in Section 8.3.1 and also with the results for sediments of S. sanguis 804 reported in Chapter 7. The nature of the plaque carbohydrate was not investigated but it is known that S. sanguis 804 synthesises glucan from sucrose.

In summary, it has been shown that the diffusion coefficient of NaF in plaque increases as the plaque carbohydrate concentration increases. In sediments of S. sanguis 804 the rates of diffusion of NaF and ¹⁴C-sucrose increase as the glucan content of the sediment increases and NaF diffuses twice as fast in cell-free glucan material as in glucan-free bacterial sediment. These results are in conflict with the widely-published postulation (see Section 2.3.1) that extracellular polysaccharides act as a barrier to diffusion in plaque.

It may be speculated that glucan formation in plaque may separate bacterial cells thus providing pathways for more rapid diffusion. It has been demonstrated that polysaccharides can form a continuous coating or capsule on the outer surface of cell walls of plaque bacteria exposed to sucrose in vivo (Saxton, 1969; Barkin, 1970) and in vitro (Lounatmaa and Meurman, 1980). The presence of large quantities of extracellular polysaccharides in plaque might therefore lead to a lower density of packing of bacteria if the plaque is free to expand in volume.

Carlsson (1967), and Carlsson and Sundström (1968) measured the microbial density in plaque, expressed as the number of cultivable organisms/mm³ of plaque, for subjects on sugar-free diets

supplemented with either sucrose or glucose mouthrinses every half-hour during the daytime. Plaque was sampled after accumulation for three days with no tooth cleaning. The microorganisms were dispersed mechanically and cultured anaerobically on blood agar plates. Four-fold more plaque was accumulated during sucrose rinsing than during glucose rinsing. However, the microbial density was two-fold greater with glucose rinsing than with sucrose rinsing. These results may be in part due to inadequate dispersal of the microorganisms, failure of some of the bacteria to grow on the culture media used, or a high proportion of non-viable cells in the plaque. However, plaque from one of the subjects was examined histologically using an electron microscope and the results confirmed that there was a lower microbial density during sucrose rinsing.

Folke et al. (1972) investigated the microbial density in plaque accumulated during four-day periods on low-sucrose or sucrose-rich diets. The microorganisms were dispersed by sonification but otherwise were cultured using the same technique as Carlsson (1967). There was no significant difference in the total amount of plaque accumulated during periods on each diet but the microbial density was about 30 per cent higher on the sucrose-rich diet than on the low-sucrose diet. Although these results may seem to be contrary to the results reported by Carlsson, it may be of significance that Folke compared low-sucrose with sucrose-rich diets whereas Carlsson compared glucose-rich with sucrose-rich diets. Further experiments are required to confirm the effect of the presence of extracellular polysaccharides on the microbial density in plaque. Due to the problems involved in determining the number

of cultivable microorganisms in plaque, it may be more reliable to investigate the plaque histologically.

It would be of value to measure diffusion coefficients for a wider range of substances in plaques of different age and carbohydrate exposure. However, the results reported in this thesis indicate that there is only a small variation in rates of diffusion in plaque from different subjects. Of the values of NaF diffusion coefficient reported in Table 8.1, all but one are within ± 25 per cent of the mean value, despite a seven-fold variation in the plaque carbohydrate concentration. A parameter of greater importance to the time for diffusion is the thickness of the plaque layer. Equation 3.4 indicates that the time required to diffuse a given distance is proportional to the (diffusion coefficient)⁻¹ but also to the distance to the second power. Thus a 25 per cent increase in plaque thickness means a 56 per cent increase in the time required to diffuse through the plaque. Numerous studies have shown that subjects on sucrose-rich diets produce more copious amounts of plaque than subjects on sucrose-free diets (e.g. Carlsson and Sundström, 1968). This is believed to be partly due to the production of extracellular polysaccharides which assist in the adhesion of bacteria and increase the amount of extracellular matrix. Therefore, the effect of extracellular polysaccharides on the thickness of the plaque layer may be of much greater significance than their effect on diffusion coefficients. There is a need for more detailed measurements of the thickness of plaque layers formed in vivo under different dietary habits.

CHAPTER 9

THE EFFECT OF DAILY MOUTHRINSES WITH 0.2 PER CENT NaF ON HUMAN DENTAL PLAQUE ACIDOGENICITY IN SITU (STEPHAN CURVE) AND PLAQUE FLUORIDE CONTENT

9.1 INTRODUCTION

The studies so far reported in this thesis concern the measurement of diffusion coefficients. The aim of the work reported in this chapter was to investigate one of the mechanisms by which mouthrinsing daily with a fluoride solution exerts a clinically demonstrable cariostatic effect. The importance of diffusion processes to this caries-preventive treatment will be discussed.

There are innumerable reports of clinically observed reductions in caries incidence caused by a variety of fluoride preparations (for a review, see Ericsson, 1977). It has been shown that mouthrinsing with fluoride solutions is an effective method of preventing caries (see review by Birkeland and Torell, 1978) e.g. daily rinses with neutral solutions of 0.05 per cent NaF and weekly rinses with neutral solutions of 0.2 per cent NaF have been shown to reduce the incidence of caries by about 40 per cent. For the study reported in this chapter, subjects were asked to rinse once daily with 15 ml of a 0.2 per cent solution of NaF (900 ppmF) for two minutes. If the early speculation on diffusion in plaque is true (see Section 2.3.1) i.e. that plaque acts as a barrier to diffusion, it would be difficult to explain

how such a brief exposure to fluoride could result in such a substantial caries reduction. However, from the measured value of diffusion coefficient of NaF in plaque reported in Chapter 4, it can be calculated that fluoride will penetrate thin layers of plaque in a few seconds and so the presence of thin layers of plaque on the teeth during mouthrinsing should not significantly reduce the amount of fluoride reaching the enamel surface.

The main mechanisms by which fluoride exerts its cariostatic effect concern its reaction with enamel. As discussed in Chapter 1, it is believed that enamel is made less soluble when fluoride ions exchange with the hydroxyl ions of hydroxyapatite to produce fluorapatite. The presence of fluoride has also been shown to encourage recrystallisation of enamel with the repair of damage caused by acid attack. However, there is also some evidence to show that fluoride may reduce the acidogenicity of dental plaque.

Several early studies showed that low concentrations of fluoride (1-10 ppm) can inhibit acid production by oral bacteria in vitro (Bibby and van Kesteren, 1940; Wright and Jenkins, 1954; Shiota, 1956; Jenkins, 1959) and fluoride concentrations of about 100 ppm and above can inhibit bacterial growth. Hamilton (1977) has reviewed some investigations designed to elucidate the methods by which fluoride regulates carbohydrate metabolism by bacteria. Fluoride inhibits several groups of bacterial enzymes including enolase, phosphatases, catalase and peroxidase but the effect on enolase seems to be the most important. Enolase is responsible for the conversion of 2-phosphoglycerate to P-enolpyruvate, one of the steps involved in the formation of pyruvate and hence lactic acid

from glucose in bacteria. Perhaps of more importance is the fact that enolase is involved in the transport of glucose across the cell wall. By inhibiting the action of enolase, fluoride can reduce the uptake of glucose by cells and so as well as reducing glycolysis, it can reduce the amount of intracellular polysaccharide formation. These polysaccharides would eventually be broken down to form acid when the exogenous supply of fermentable carbohydrates was depleted. The degree of inhibition exerted by fluoride is greater at low pH values due to an increased permeability of cells to fluoride.

There are few studies to show whether or not fluoride can significantly reduce acid production in dental plaque. Jenkins, Edgar and Ferguson (1969) and Edgar, Jenkins and Tatevossian (1970) reported a slightly smaller fall in pH when plaque from subjects living in areas with fluoridated water supplies was incubated with sucrose in vitro or in vivo, compared with plaque from subjects living in a non-fluoridated area. Woolley and Rickles (1971) applied 2.0 per cent solutions of NaF to plaque-covered teeth for four minutes and subsequently monitored the fall in plaque pH in vivo following a sucrose mouthrinse (Stephan curve). The pH fall was significantly reduced when measured up to eight hours after the NaF application, but had returned to normal within three or four days.

Geddes (1974) investigated the effect of daily mouth-rinsing with 0.2 per cent solutions of NaF on plaque acidogenicity in vivo, which was assessed by measuring Stephan curves. Fluoride rinsing was shown to result in a small rise in the minimum pH

value of the Stephan curve and a large increase in the plaque fluoride concentration. The experiment was slightly complicated by the fact that most of the subjects were participating in an experimental caries study (Edgar *et al.*, 1978 a, b; Geddes *et al.*, 1978). Following a baseline Stephan curve measurement, these subjects abstained from all forms of oral hygiene for 18 days, before commencing a two month period of fluoride rinsing, at the end of which a second Stephan curve measurement was made. Also, these subjects were resident in an area (Newcastle upon Tyne) with a fluoridated tap-water supply (1.0 ppm F).

The aim of the work reported in this chapter was to repeat this study by Geddes in Glasgow, a non-fluoridated area with less than 0.05 ppm F in the tap-water supply, omitting the experimental caries phase. This work was conducted jointly with Dr. Geddes.

9.2 METHODS

9.2.1 Experimental Protocol

Twenty young adult subjects (sixteen male, four female) were studied in this longitudinal design of experiment. The subjects were all members of staff (clerical, technical and professional) of the Glasgow Dental Hospital and School, with controlled, moderate levels of caries. The subjects were given a supply of a non-fluoridated toothpaste (Sensodyne; Stafford-Miller Ltd., Hatfield, Herts., England) and were receiving no other forms of fluoride therapy. The fluoride content of Sensodyne toothpaste was measured using Orion fluoride-specific electrodes, after

mixing 0.2 g of toothpaste with 10 ml of TISAB buffer (Orion, 1977). The mean fluoride concentration in the toothpaste, \pm standard deviation, from eight measurements was (33 ± 2) ppm. By comparison, a popular brand of fluoride-supplemented toothpaste (Crest; Procter and Gamble Ltd., Newcastle upon Tyne, England) contains 970 ppm fluoride (as stannous fluoride).

At least one week after adopting the low-fluoride toothpaste, each subject accumulated plaque on three separate occasions by refraining from tooth-cleaning for periods of 24 h. On the first occasion, a 'practice' Stephan curve was measured (details to follow). This served to accustom the investigator who was to collect the plaque (Dr. Geddes) with the distribution of plaque accumulation for each subject and also acquainted the subjects to the experimental technique. This was considered necessary because the timing of the plaque sampling was to be strictly standardised. The plaque was removed on the second occasion for fluoride analysis (details to follow) and on the third occasion, the Stephan curve measurement was repeated and used as a control measurement for the experiment.

The subjects were then supplied with 500 ml of a 0.2 per cent (w/v) aqueous solution of NaF (pH 6.6) contained in a polythene screw-cap bottle and some polypropylene measuring cups. The subjects were asked to rinse with 15 ml of the fluoride solution for two minutes each evening after tooth-cleaning before retiring to bed. The rinse was expectorated after two minutes and the subjects were instructed not to rinse with water immediately after the fluoride rinse. After a month of daily fluoride rinsing,

further measurements were made of plaque fluoride concentrations and Stephan curves. Plaque was accumulated for 24 h as before, and fluoride rinsing, but not tooth-cleaning, was performed on the evening prior to measurement. The effect of fluoride rinsing was assessed for each subject by comparing the measurements made during the rinsing period with the control measurements.

For the experiments described above, the Stephan curves were measured between eight and twelve hours after the fluoride rinse. A subsidiary experiment was performed to assess the effect of incorporating 0.2 per cent NaF in the 20 per cent sucrose solution used to induce the Stephan curve. Four subjects were used for these measurements, which were made at least six months after the subjects had finished the fluoride rinsing phase and had returned to their normal oral hygiene habits. The measurements were again made on plaque accumulated for 24 h.

9.2.2 Measurement of Plaque Acidogenicity (Stephan Curve).

Plaque acidogenicity was assessed using the modification by Geddes (1974) of the technique described by Englander, Carter and Fosdick (1956) and by Frostell (1969; 1970). Measurements were made mid-morning, at a time standardised for each subject, at least two hours after the subject's last food or drink consumption and (for the rinse phase) between eight and twelve hours after the last fluoride rinse. Resting plaque pH was measured by scraping a small amount of plaque from each accessible tooth surface using a smooth, nickel-plated micro-spatula. The speed of sampling was paced to occupy exactly one minute. This pooled plaque sample of

approximately 1 mg, was placed in the bowl of a 'one-drop' glass pH electrode (Beckman-R11C Ltd., Glenrothes, Fife, Scotland) together with 10 μ l of double-distilled, deionised water. A Beckman fibre-junction calomel reference electrode was positioned to make electrical contact and pH was read on a pH meter (Model pH 11/B, Chiltern Scientific Instrumentation Ltd., Penn St. Village, Bucks., England) to 0.01 pH units, the reading being taken exactly one minute after sampling. The subject then rinsed for 30 s with 10 ml of a 20 per cent (w/v) sucrose solution, which was then expectorated. Plaque pH was measured by sampling plaque 5, 7, 10, 12, 15 and 20 min after the start of the sucrose rinse. Plaque sampling on each occasion was paced to last one minute, the plaque was then immediately placed onto the pH electrode, and pH was recorded after a further minute. This technique of measuring Stephan curves, by which the incubation of sucrose occurs in situ and the pH is measured in vitro has been shown to be reproducible and highly characteristic of the individual subject under study (Frostell, 1970; Geddes, 1974; Edgar, 1976).

Two parameters which can be calculated from these Stephan curves are of particular value in the assessment of plaque acidogenicity. These are, firstly, the lowest pH value recorded (pH min) and secondly, a parameter ($p\Sigma\Delta cH$) which gives a measurement of the total area of the Stephan curve and which therefore reflects the total acid production. Edgar (1976) described the calculation of $p\Sigma\Delta cH$. The pH values are firstly converted to hydrogen ion concentrations (cH) using the relation $pH = -\log_{10} cH$, or $cH = 10^{-pH}$. The resting cH value (recorded immediately prior to the sucrose rinse) is subtracted from each of the six subsequent cH values to give

values of ΔCH , which are then summed to give $\Sigma\Delta\text{CH}$. Finally, the negative of the logarithm to the base 10 of $\Sigma\Delta\text{CH}$, gives $p\Sigma\Delta\text{CH}$.

It should be noted that, as for pH, low values of $p\Sigma\Delta\text{CH}$ correspond to high levels of acid. Edgar (1976) showed that $p\Sigma\Delta\text{CH}$ conforms to a Gaussian distribution and can therefore be analysed using parametric statistics.

9.2.3 Plaque Fluoride Analysis.

After 24 h accumulation, plaque was removed from all accessible tooth surfaces using a smooth nickel-plated spatula, at least two hours after the subject's last food or drink and (during the rinsing phase) between eight and twelve hours after the last fluoride rinse. Total plaque fluoride was assayed using Edgar's modification (personal communication, 1976) of the hot acid extraction technique of Birkeland (1970). After removal from the mouth, 10-15 mg of plaque were placed in a polypropylene, conical-shaped test tube, of capacity 1.5 ml (Walter Sarstedt U.K. Ltd., Leicester, England). The weight of the plaque was determined to 0.01 mg on a balance (Type H16, Mettler Ltd.) and 0.1 ml of 50 per cent sulphuric acid was added to the plaque. The test tube was then placed upright in a 30 ml polystyrene-polypropylene Universal tube (product code no. 128, Sterilin Ltd., Teddington, Middlesex, England) in the base of which was 0.5 ml of 1N NaOH and 1.5 ml of 2 M sodium acetate-acetic acid buffer, pH 5.2. A layer of silicone high vacuum grease (Edwards Vacuum Components Ltd., Crawley, Sussex, England) was applied to the cap of the Universal tube to ensure a gas-tight seal, and the tube was placed upright in an incubator at 60°C and left overnight. Next morning, the Universal tube was shaken to mix the acid, alkali and buffer. Fluoride standards, using NaF, were

prepared by the same method. Samples were then analysed for fluoride using an Orion fluoride-specific electrode and separate reference electrode, connected to an Orion model 701A digital mV meter. The fluoride concentrations were all expressed as parts per million of the plaque wet weight. All of the chemical reagents used in this assay were AnalaR grade (B.D.H. Ltd.).

9.3 RESULTS

All of the subjects displayed a classical 'Stephan curve' response to the sucrose rinse, although there was considerable inter-subject variability regarding the minimum pH value recorded and the time after the sucrose rinse at which this occurred. The values of plaque pH, plaque F and $p\Sigma\Delta\text{CH}$ for each subject prior to (control) and during (test) fluoride rinsing are listed in Table 9.1, together with values of $\Delta\text{pH min}$, the difference in the pH min between test and control measurements.

Statistical analysis of the changes in resting pH, pH min, $p\Sigma\Delta\text{CH}$ and plaque F with fluoride rinsing is detailed in Table 9.2. Before rinsing, plaque F values ranged from 0.8 to 10.6 ppm with a mean of 4.7 ppm. During rinsing, plaque F concentration increased to between 21.9 and 151 ppm, with a mean of 58.3 ppm. This increase was highly significant ($p < 0.001$) when tested using the paired t-test (Snedecor and Cochran, 1967). The mean resting pH levels for the control and test phases were 6.80 and 6.79 respectively, and were not statistically significantly different. The mean value of pH min was raised by 0.12 pH units from 5.64 to 5.76 with fluoride rinsing, but this increase just failed to be of statistical significance ($0.1 > p > 0.05$, paired t-test).

Table 9.1 Plaque pH (Stephan curve) and plaque fluoride measured for 20 subjects prior to (control) and during (test) a fluoride mouthrinsing regimen.

Δ pH min is the increment in the minimum pH value between the control and test curves, and $p\Sigma\Delta$ CH is a measure of the area of the curve (see text for details).

Subject	resting	plaque pH (minutes after sucrose rinse)							Δ pH min	$p\Sigma\Delta$ CH	plaque fluoride (ppm)
		5	7	10	12	15	20				
CM	control	6.69	6.16	6.23	5.69	6.01	6.09	6.80		5.393	10.6
	test	6.65	6.05	6.05	5.92	6.13	6.02	6.31	+0.23	5.417	41.4
RD	control	6.79	5.36	5.49	5.79	5.81	5.97	6.47		4.950	7.2
	test	6.92	5.58	5.83	5.82	6.03	6.32	6.31	+0.22	5.167	92.5
KY	control	6.89	5.72	5.81	5.17	5.98	6.12	6.43		4.935	7.8
	test	6.72	5.28	5.37	6.05	6.20	6.27	6.57	+0.11	4.971	37.7
WB	control	6.70	5.48	5.39	5.20	5.43	5.73	5.58		4.684	1.5
	test	6.40	5.42	5.43	5.27	5.36	5.31	5.30	+0.07	4.606	44.9
LJ	control	6.77	6.38	5.83	5.60	5.80	5.64	5.90		5.069	1.6
	test	6.92	6.10	6.09	6.05	5.49	5.88	5.93	-0.11	5.125	25.8
WD	control	6.98	5.70	5.74	5.69	5.93	6.02	6.42		5.111	3.1
	test	6.65	6.20	5.97	6.02	6.07	6.06	6.11	+0.28	5.419	21.9
DWR	control	6.61	5.82	5.59	5.80	5.72	5.49	5.69		4.944	3.2
	test	6.60	6.37	6.02	6.10	6.24	6.12	6.29	+0.53	5.599	38.0

Cont.

Table 9.1 (cont.)

Subject		resting	plaque pH (minutes after sucrose rinse)							Δ pH min	$\sum \Delta$ pH	plaque fluoride (ppm)
			5	7	10	12	15	20	20			
JM	control	6.78	6.06	6.17	5.58	6.20	6.24	6.40		5.320	9.9	
	test	6.84	6.22	6.36	6.41	6.58	6.58	6.70	+0.64	5.891	23.3	
PL	control	6.82	6.69	6.30	6.08	6.21	6.31	6.30		5.650	4.1	
	test	6.73	6.44	6.02	6.18	6.10	6.39	6.18	-0.06	5.565	85.8	
DM	control	6.89	6.10	6.22	6.21	6.02	6.27	6.20		5.473	4.1	
	test	7.00	5.90	5.76	5.89	5.70	6.00	6.02	-0.32	5.117	95.7	
JB	control	6.87	5.72	5.53	5.79	5.50	5.66	5.70		4.886	1.6	
	test	6.80	6.03	6.20	5.93	5.88	6.02	6.25	+0.38	5.335	33.9	
GW	control	6.63	5.72	5.55	5.88	6.30	5.90	5.90		5.116	7.2	
	test	6.60	5.92	5.77	5.27	5.85	5.40	5.77	-0.28	4.858	125	
DE	control	7.03	5.92	6.07	6.09	6.10	6.31	6.40		5.399	5.3	
	test	7.10	6.10	6.45	6.20	6.32	6.57	6.50	+0.18	5.626	22.2	
LS	control	6.93	6.21	5.95	6.38	6.28	6.58	6.71		5.614	9.2	
	test	6.88	6.05	5.90	6.07	6.10	6.22	6.38	-0.05	5.395	89.5	
JT	control	6.28	5.90	6.08	5.05	5.70	5.40	5.83		4.815	7.0	
	test	6.32	5.58	5.40	5.41	5.30	5.10	5.35	+0.05	4.601	53.1	
MS	control	7.05	6.30	6.18	6.50	6.50	6.50	6.58		5.735	1.2	
	test	7.03	6.07	6.30	6.60	6.45	6.57	6.66	-0.11	5.724	101	

Cont.

Table 9.1 (cont.)

Subject	resting	plaque pH (minutes after sucrose rinse)					Δ pH min	$p\sum\Delta$ CH	plaque fluoride (ppm)
		5	7	10	12	15			
SD	6.80	5.50	5.45	5.47	5.51	5.82	6.00	4.831	1.2
	6.97	5.90	6.12	5.90	5.80	6.09	6.28	5.255	26.9
RR	6.55	5.32	5.49	5.49	5.92	5.62	5.80	4.831	2.8
	6.97	5.66	5.80	6.10	6.00	6.40	6.53	5.250	151
DL	6.94	6.12	6.01	6.31	6.00	6.35	6.60	5.490	4.2
	6.79	6.50	6.20	6.05	6.21	6.62	6.60	5.705	26.3
MW	6.96	6.13	6.25	6.10	6.08	6.46	6.60	5.542	0.8
	6.97	6.51	6.00	5.80	6.40	6.41	6.59	5.482	30.8

Table 9.2 Mean values of resting pH, pH min, $p\Sigma\Delta\text{cH}$ and plaque F, measured for 20 subjects prior to (control) and during (test) a fluoride mouthrinsing regimen. Figures in brackets are standard deviations. Statistical analysis was performed using the paired t-test.

	resting pH	pH min	$p\Sigma\Delta\text{cH}$	plaque F (ppm)
control	6.80 (0.19)	5.64 (0.34)	5.19 (0.33)	4.7 (3.2)
test	6.79 (0.21)	5.76 (0.33)	5.31 (0.36)	58.3 (38.9)
t	0.133 p > 0.5	1.822 0.1 > p > 0.05	1.795 0.1 > p > 0.05	6.178 p < 0.001*

*statistically significant difference between test and control.

Similarly, the increase of $p\Sigma\Delta\text{CH}$ from a mean value of 5.19 to 5.31 with fluoride rinsing, which indicates a reduction in acid production, just failed to reach statistical significance ($0.1 > p > 0.05$, paired t-test).

A scatter diagram was plotted (Fig. 9.1) to show whether the pH min increased or decreased with fluoride rinsing, plotted against the pH min of the control Stephan curve. Thirteen of the twenty subjects displayed a raised pH min with fluoride rinsing. The subjects were divided into two groups. Thirteen subjects gave a pH min below 5.7 during the control phase, and all but two of these subjects had a raised pH min with fluoride rinsing. Seven subjects gave a pH min of above 5.9 for the control curve, two giving an increase and five a decrease in pH min with fluoride rinsing.

The data from 13 subjects in the low pH group were re-analysed (Table 9.3). The mean resting pH both before and during fluoride rinsing was 6.72, but the mean pH min increased by 0.22 units, from 5.43 to 5.65, with fluoride rinsing. This increase is statistically significant at the 0.01 level (paired t-test). There was also a statistically significant ($p < 0.05$, paired t-test) increase (less acid) in the mean $p\Sigma\Delta\text{CH}$, from 4.99 to 5.19, with fluoride rinsing. Plaque F concentration increased on average eleven-fold, from 5.0 to 55.0 ppm with fluoride rinsing.

The Stephan curve measurements for the four subjects who were given 0.2 per cent NaF in the 20 per cent sucrose mouthrinse used to induce the Stephan curve, are listed in Table 9.4. Means of these pH measurements at each sampling time were calculated, as

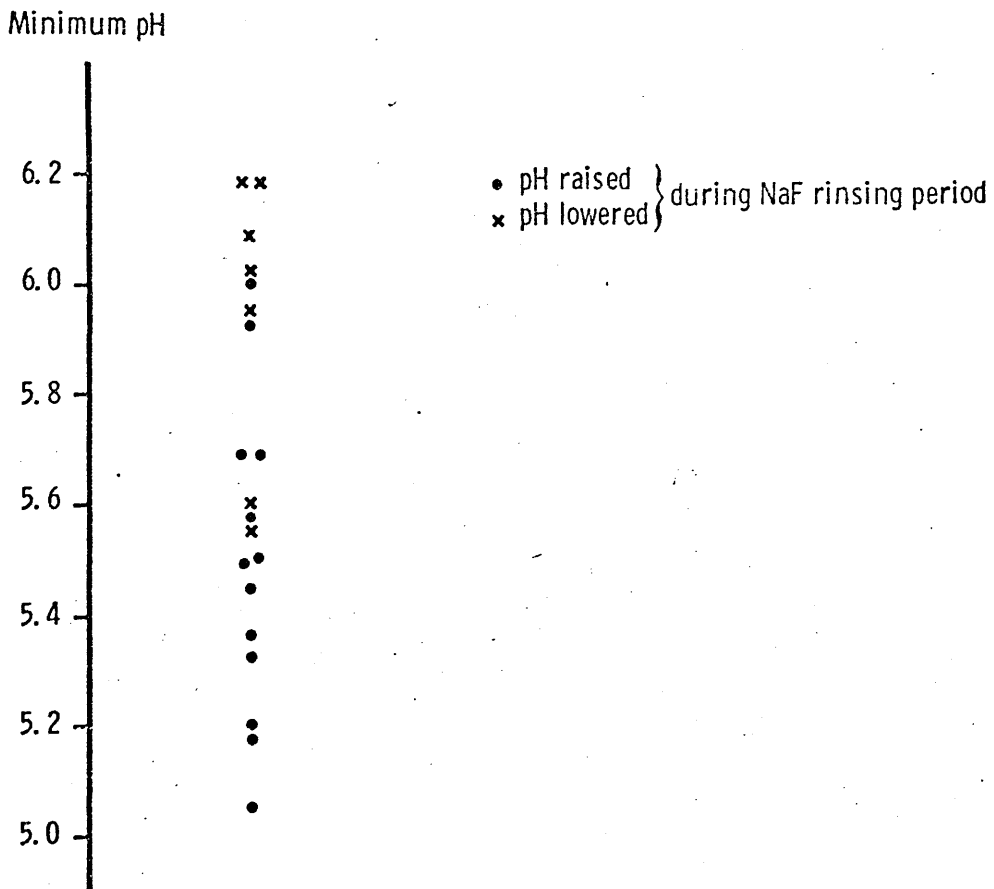


Fig. 9.1 Scatter diagram indicating whether the Stephan curve pH min was raised or lowered by fluoride rinsing, plotted as a function of the pH min value.

Table 9.3 Mean values of resting pH, pH min, $p\Sigma\Delta\text{cH}$ and plaque F, measured for 13 subjects prior to (control) and during (test) a fluoride mouthrinsing regimen. For each subject, the pH min for the control measurement was less than 5.7.

Figures in brackets are standard deviations.
Statistical analysis was performed using the paired t-test.

	resting pH	pH min	$p\Sigma\Delta\text{cH}$	plaque F (ppm)
control	6.72 (0.18)	5.43 (0.20)	4.99 (0.20)	5.0 (3.4)
test	6.72 (0.21)	5.65 (0.35)	5.19 (0.37)	55.0 (41.4)
t	0.027 p > 0.5	3.173 p < 0.01*	2.448 p < 0.05*	4.656 p < 0.001*

*statistically significant difference between test and control.

Table 9.4 Stephan curve measurements for four subjects following a 30 s mouthrinse with 15 ml of a 20 per cent sucrose solution containing 0.2 per cent NaF, and the corresponding means of the pH measurements for these four subjects for the test and control periods. Figures in brackets are standard deviations.

Subject	resting	plaque pH					
		5	7	10 (minutes after sucrose rinse)	12	15	20
CM	7.00	6.95	6.96	6.81	6.80	6.30	6.50
WB	7.02	6.78	6.80	6.33	6.30	6.20	6.25
LJ	6.83	6.79	6.79	6.59	6.48	6.48	6.57
WD	6.78	6.71	6.48	6.09	6.39	6.00	6.24
means (S.D.)	6.91 (0.12)	6.81 (0.10)	6.76 (0.20)	6.46 (0.31)	5.49 (0.22)	6.25 (0.20)	6.39 (0.17)
corresponding means for:-							
(a) control	6.79 (0.13)	5.93 (0.41)	5.80 (0.35)	5.55 (0.23)	5.79 (0.26)	5.87 (0.22)	6.18 (0.54)
(b) test	6.66 (0.21)	5.94 (0.35)	5.89 (0.31)	5.82 (0.37)	5.76 (0.39)	5.82 (0.35)	5.91 (0.44)

well as for the control and test Stephan curves for these subjects. These mean curves are plotted in Fig. 9.2. The pH min values for the control, test and 'fluoride-with-sucrose' curves are 5.55, 5.76 and 6.25, respectively. The difference in pH min between the control and test curves is not statistically significant ($p > 0.2$). However the rise in pH min when fluoride was included with the sucrose is statistically significant ($p < 0.005$) when compared to the control curve, and just fails to reach statistical significance ($0.1 > p > 0.05$) when compared to the test curve. Because of the low number of subjects, and the length of the time interval between the control and the 'fluoride-with-sucrose' measurements, Student's t-test was used for these comparisons, in preference to the paired t-test.

The parameters resting pH, pH min, $p\Sigma\Delta\text{cH}$ and plaque F for all 20 subjects, were examined for correlation using linear regression analysis, for both control and test measurements (Table 9.5a). Increments in plaque F, pH min and $p\Sigma\Delta\text{cH}$ with fluoride rinsing were also examined for correlation (Table 9.5b). The results show that both prior to, and during fluoride rinsing, pH min was highly correlated with $p\Sigma\Delta\text{cH}$ ($p < 0.001$) and resting pH was also correlated with $p\Sigma\Delta\text{cH}$ ($p < 0.05$). There was a high degree of correlation between resting pH and pH min prior to fluoride rinsing ($p < 0.001$), but this correlation was weaker during rinsing ($p < 0.05$). No significant correlation was found between plaque F and any of the other three parameters, either prior to, or during, fluoride rinsing. Likewise, the increment in plaque F was not significantly correlated with the increments in pH min or

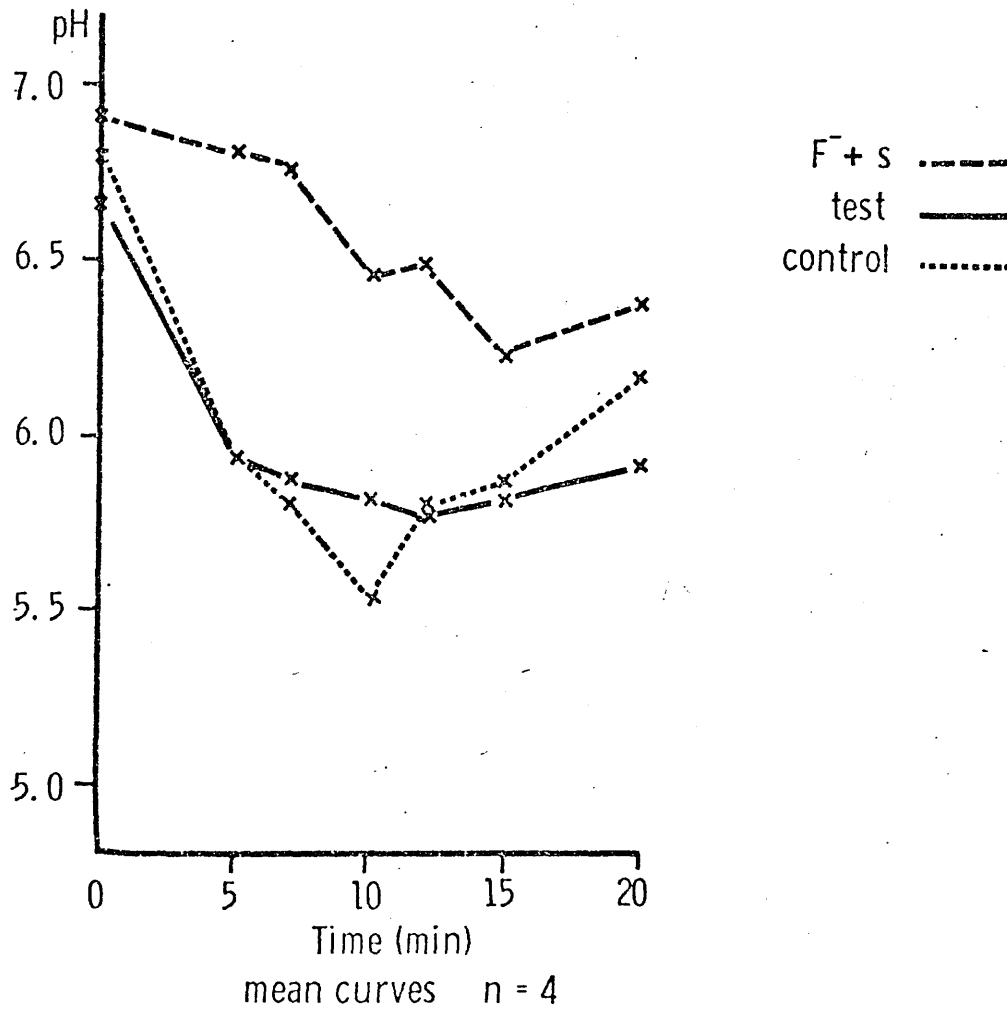


Fig. 9.2 Stephan curves prepared by averaging the pH measurements made on four subjects (CM, WB, LJ and WD) for the control (prior to fluoride rinsing) and test (with fluoride rinsing) periods, and with 0.2 per cent NaF included in the sucrose mouthrinse ($F^- + s$).

Table 9.5 Correlation matrices for plaque pH and fluoride parameters measured for 20 subjects prior to (control) and during (test) a fluoride mouthrinsing regimen.

(a)		pH min	p $\Sigma\Delta$ cH	plaque F
control	resting pH	***	*	N.S.
	pH min		***	N.S.
	p $\Sigma\Delta$ cH			N.S.
test	resting pH	*	*	N.S.
	pH min		***	N.S.
	p $\Sigma\Delta$ cH			N.S.

(b) Increment between control and test measurements

	Δ pH min	Δ (p $\Sigma\Delta$ cH)
Δ plaque F	N.S.	N.S.
Δ pH min		***

N.S. not significant

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

$p\Sigma\Delta\text{CH}$. The increment in pH min was highly significantly correlated with the increment in $p\Sigma\Delta\text{CH}$ ($p < 0.001$).

These correlation tests were repeated using only the 13 subjects in the low pH group (Table 9.6). Again, pH min was highly significantly correlated with $p\Sigma\Delta\text{CH}$ prior to ($p < 0.01$) and during ($p < 0.001$) fluoride rinsing, and the increment in pH min was highly correlated with the increment in $p\Sigma\Delta\text{CH}$ ($p < 0.001$). The only correlation with resting pH was by $p\Sigma\Delta\text{CH}$ during the rinse phase ($p < 0.01$) and plaque F was found to have a significant correlation to $p\Sigma\Delta\text{CH}$ prior to rinsing ($p < 0.01$).

9.4 DISCUSSION

Fluoride mouthrinsing resulted in a large increase in plaque fluoride concentrations. The fluoride concentrations measured prior to rinsing (mean value 4.7 ppm of plaque wet weight) and during rinsing (mean value 58.3 ppm of plaque wet weight) were similar to the values reported by Geddes (1974), despite the difference in fluoride content of the respective tap-water supplies. However, it is known (Edgar, 1973; Jenkins and Edgar, 1977) that fluoride is present in plaque in different forms. The hot acid extraction technique used for the samples reported herein measures the total plaque fluoride content, whereas the cold acid extraction technique used by Geddes (1974) measures only the fraction of fluoride which is loosely bound within plaque. The plaque fluoride contents measured prior to fluoride rinsing are similar to those reported by Jenkins and Edgar (1977) for five-year old schoolchildren, but lower than those reported by Birkeland

Table 9.6 Correlation matrices for plaque pH and fluoride parameters measured for 13 subjects prior to (control) and during (test) a fluoride mouthrinsing regimen. For each subject, the pH min of the control Stephan curve was less than 5.7.

(a)		pH min	$p\Sigma\Delta\text{cH}$	plaque F
control	resting pH	0	N.S.	N.S.
	pH min		**	N.S.
	$p\Sigma\Delta\text{cH}$			**
test	resting pH	N.S.	**	N.S.
	pH min		***	N.S.
	$p\Sigma\Delta\text{cH}$			N.S.

(b) Increments between control and test measurements

	Δ pH min	$\Delta(p\Sigma\Delta\text{cH})$
Δ plaque F	N.S.	N.S.
Δ pH min		***

N.S. not significant

0 $0.1 > p > 0.05$

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

(1970), Singer et al. (1970) and Edgar (1973), hot acid extraction being used in each case. These differences may be related to variations in fluoride concentration in the domestic water supplies and in the use of fluoride-containing dentifrices. The plaque fluoride concentrations measured during the fluoride rinsing phase are similar to the concentrations reported by Birkeland, Jorkjend and von der Fehr (1971) for schoolchildren rinsing once weekly with 0.2 per cent NaF solutions.

At the time of fluoride rinsing on the evening prior to plaque sampling, the subjects had not cleaned their teeth for a period of about twelve hours. The observed increase in plaque fluoride concentration, in plaque collected between eight and twelve hours after the fluoride rinse, confirms that the fluoride could rapidly diffuse through the thin layers of plaque to reach the enamel surface. It may be that this fluoride was strongly bound within the plaque at the time of the rinse. Tatevossian (1978) reported elevated fluoride concentrations in plaque two hours, although not three hours, after a one minute mouthrinse with 20 mM NaF (380 ppm F). Alternatively, some of the fluoride may have been incorporated into the plaque on being slowly leached from the enamel, in accordance with the hypothesis made by Hardwick (1970). By retaining high concentrations of fluoride, the plaque may prolong the length of time during which the tooth surface is subject to the effects of the rinse.

The pH min of the group of twenty subjects reported in this thesis was raised during fluoride rinsing by an average of 0.12 units, but this increase just failed to reach statistical significance

($0.1 > p > 0.05$). In contrast, the average rise in pH min of 0.14 units reported by Geddes (1974) was statistically significant at the 0.05 level. Closer examination reveals that for seven of the subjects used in the study reported herein, the pH during the Stephan curve was never lower than 5.9. These subjects probably have a low risk of caries because, as discussed in Chapter 1, the critical pH of plaque at which enamel begins to dissolve seems to be about 5.5. The subjects are therefore likely to receive little benefit from a treatment designed to reduce plaque acid production. It also seems likely that a reduction in plaque acid production would more easily be detected using subjects who produce large amounts of acid in their plaque. This was found to be true by looking at the remaining group of thirteen subjects whose pH min during the control Stephan curve was lower than 5.7. For these subjects there was a statistically significant ($p < 0.01$) increase in pH min, on average by 0.22 units, with fluoride rinsing. There was also a significant increase (less acid) in $p\Sigma\Delta\text{CH}$ ($p < 0.05$). This effect was measured between eight and twelve hours after the last fluoride rinse. When fluoride was included in the sucrose rinse used to induce the Stephan curve, the effect on plaque pH was more pronounced, and the plaque pH stayed above 6.0 (Table 9.4).

Although daily fluoride rinsing has been found to result in a small raising of the pH min, such an effect could be of clinical significance. For some subjects a small raising of the pH min may be sufficient to prevent the plaque pH from falling below the critical value for enamel dissolution. It should also reduce the length of time for which enamel is exposed to harmful concentrations of acid, and any reduction in plaque acid production

can only be of benefit. To optimise the effect of fluoride rinses on plaque acidogenicity would require an increase in the fluoride concentration or the frequency of rinsing, or both. Alternatively, fluoride could be included with sugar. This would not be recommended as a caries-preventive measure for the general public because the total dietary intake of fluoride could not be adequately controlled. The effect of fluoride on plaque acidogenicity would also have to be balanced with the more important effect of fluoride on enamel solubility and remineralisation.

The correlation analyses (Tables 9.5 and 9.6) reveal that pH min is highly correlated with $p\sum\Delta\text{cH}$, supporting the viewpoint of Edgar (1976) that both of these parameters are of value in the analysis of Stephan curves in screening tests of the cariogenicity of dietary components. Resting pH correlated with pH min and $p\sum\Delta\text{cH}$ for the group of twenty subjects, again in agreement with Edgar (1976), but these correlations were in general weaker for the group of thirteen subjects who produced large amounts of acid. The most puzzling finding was that the only significant correlation with plaque fluoride concentration was by $p\sum\Delta\text{cH}$ for the group of thirteen subjects prior to fluoride rinsing. Geddes (1974) found that the resting pH during fluoride rinsing was the only parameter to correlate with plaque fluoride concentration. Furthermore, for the study reported herein, the increase in plaque fluoride concentrations with rinsing did not correlate with the changes in pH min or $p\sum\Delta\text{cH}$. However, fluoride can occur in plaque in both strongly and loosely bound forms and as free ions (Jenkins and Edgar, 1977). The total plaque fluoride is measured by the hot acid extraction technique. As it is not, as yet, known which

form of fluoride is capable of inhibiting acid production, a correlation between plaque fluoride and plaque pH might only be determined by examining the different forms of fluoride in plaque.

To summarise, daily rinsing with 0.2 per cent NaF solutions resulted in a substantial increase in the plaque fluoride content and a small rise in the minimum pH of the Stephan curve. This pH rise was greater for subjects who produced large amounts of plaque acid. There was a marked reduction in plaque acid production when 0.2 per cent NaF was included in the sucrose rinse used to induce the Stephan curve. It is recommended that only subjects whose plaque pH falls below 5.7 during the Stephan curve should be selected for studies designed to test plaque acidogenicity. That the above effects were observed despite the brevity of each fluoride rinse (two minutes), suggests that thin layers of plaque were not able to form a barrier to the diffusion of fluoride to prevent its access to the tooth surface.

CHAPTER 10

CONCLUDING DISCUSSION

In 1976, prior to the start of work for this thesis, it was generally believed that dental plaque acted as a barrier to diffusion (see Chapter 2). Although it was accepted that sugars could rapidly penetrate into plaque, it was thought that acid produced by metabolism of the sugars by plaque bacteria would be trapped at the tooth surface and that the rate of penetration of neutralising agents from saliva into plaque would be restricted. Several workers postulated that extracellular polysaccharides regulated the rates of diffusion in plaque. However, these opinions were based partly on qualitative information and partly on speculation. The most common fault of previous studies was a failure to isolate the diffusion process from the effects of metabolic or chemical interactions. The aim of the work reported in this thesis was to quantify rates of diffusion in dental plaque. In this chapter the results of this thesis will be compared with those of other workers and their significance in relation to dental caries will be discussed.

Table 10.1 summarises the diffusion coefficients in dental plaque at 37°C reported in Chapters 3 to 6. Listed in column three of this table are calculated values of the halving-times, $T_{\frac{1}{2}}$, for a 100µm thick layer of plaque and in column four are values of the diffusivity of each substance in plaque. This diffusivity is

Table 10.1 Summary of the measurements of diffusion coefficients and diffusivities in dental plaque at 37°C from Chapters 3, 4, 5 and 6 of this thesis, with calculated values of $T_{\frac{1}{2}}$ for a 100 μm plaque layer.

Diffusing substance	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$	$T_{\frac{1}{2}}$ for 100 μm plaque layer (s)	Diffusivity
Xenon	7.2 ± 0.6	3.9	0.46
O_2 *	14.7	1.9	-
CO_2 *	12.5	2.2	-
NaF	4.2 ± 0.5	6.7	0.23
Sucrose**	3.0 ± 0.3	9.4	0.43
Xylitol	2.3 ± 0.2	12.2	-
Sodium acetate**	5.0 ± 0.7	5.6	0.31
Sodium lactate**	4.8 ± 0.3	5.9	0.31

\pm standard deviations

* estimated using Graham's law

** glutaraldehyde-fixed plaque

defined as the ratio of the diffusion coefficient in plaque to the diffusion coefficient in water and values were calculated using the diffusion coefficients for water listed in the Appendix. The diffusivity is a measure of the extent to which plaque restricts the diffusion of a substance.

The diffusion coefficients in plaque are between two and five orders of magnitude greater than the largest values reported for diffusion coefficients in enamel (see review in Chapter 2). The diffusivity values range from 0.23 to 0.46 so that the rates of diffusion in plaque are between a quarter and a half of the rates of diffusion in water. The calculated $T_{\frac{1}{2}}$ values for a 100 μm layer of plaque range from 2 to 12 s and these results all show that diffusion in plaque is a rapid process.

The study of diffusion of xenon in plaque is of value for two reasons. Firstly, because the xenon atom is chemically inert and electrically neutral, it should reflect the ability of plaque to act as a physical barrier to diffusion, either due to the presence of solid material or because of narrow channels for diffusion. Secondly, using Graham's law of diffusion, values can be calculated for the diffusion coefficients in plaque of gases of metabolic importance, such as oxygen and carbon dioxide. The diffusivity of xenon in plaque was 0.46 i.e. diffusion of xenon in plaque is about half as fast as diffusion in water. This difference can be explained by the presence of insoluble solids in plaque causing an increased path length for diffusion and also because plaque fluid is more viscous than water. The $T_{\frac{1}{2}}$ of 3.9 s for xenon in a 100 μm layer of plaque conflicts with the suggestion that

plaque acts as a diffusion barrier. Likewise, oxygen and carbon dioxide will diffuse very rapidly in plaque in the absence of metabolic interaction, with $T_{\frac{1}{2}}$ values of 1.9 and 2.2 s respectively for a 100 μm layer of plaque. These diffusion coefficients are of importance with regard to the composition of the bacterial flora of plaque. The maximum depth to which oxygen will penetrate a layer of plaque depends on the oxygen diffusion coefficient and the rate of oxygen consumption by bacterial respiration. Consequently, the thickness of the plaque layer will, in part, determine the proportions of aerobic and anaerobic bacteria in the plaque. This may be related to the cariogenicity of the plaque because the types of acid produced by glycolysis depend on the oxygen tension and on the types of bacteria present. Further measurements of the rate of oxygen consumption by plaque are required for accurate estimation of the oxygen-depletion depth.

It was important to measure diffusion coefficients of ions in plaque, and fluoride, because of its proven cariostatic properties, was chosen for particular study. The diffusivity of NaF in plaque was 0.23, only half of the diffusivity of xenon. It seems likely that the diffusion of Na and F ions is retarded by a small amount due to electrostatic interaction with cell walls and highly charged constituents of plaque matrix. Despite the four-fold difference in the rates of diffusion of NaF in plaque and water, the $T_{\frac{1}{2}}$ for NaF diffusing in a 100 μm layer of plaque is only 6.7 s. Other simple electrolytes, such as KCl, NaCl, KI, HCl and NH_4Cl , diffuse in water as fast or even faster than NaF (see Appendix) and it is therefore likely that most simple electrolytes diffuse rapidly in plaque.

The results suggest that fluoride can rapidly penetrate thin layers of plaque on the tooth surface, although measurements should be made of the diffusivity of other forms of fluoride used clinically, such as stannous fluoride, amine fluoride and sodium monofluorophosphate. Thus, a thorough cleaning and polishing of the teeth prior to topical application of fluorides may not be essential for effective penetration of enamel by fluoride. Instead it may be sufficient for the patient to brush his own teeth prior to the fluoride application. Several studies have shown that a coating of plaque does not significantly reduce the amount of fluoride taken up by enamel (Tinanoff, Wei and Parkins, 1974; 1975; Kirkegaard *et al.*, 1975; Joyston-Bechal, Duckworth and Braden, 1976; Bruun and Stoltze, 1976) although in some cases the nature of the reaction between the fluoride and the enamel was modified in the presence of plaque. Tatevossian (1978) showed that plaque fluoride concentrations were significantly higher than resting values 2 h after a 1 min rinse with 0.2 M NaF solution, hence the presence of plaque may prolong the time for interaction between fluoride and enamel. The caries-preventive effect of fluoride may not be directly proportional to the amount of fluoride taken up by enamel. Therefore, an investigation is in progress in Glasgow under the directorship of Dr K.W. Stephen in which one of the aims is to monitor the caries increment in schoolchildren treated with topical fluoride preparations with and without prior cleaning of the teeth.

Diffusion coefficients in plaque for sucrose, acetate and lactate could only be measured by preventing metabolic activity within the plaque. This was achieved by fixation of the plaque in

glutaraldehyde solution, a treatment which was shown to have only a small effect on the rates of diffusion in plaque of NaF and xylitol. The diffusivity of sucrose in plaque was 0.43 (Table 10.1) which is close to the value for xenon, and further suggests that uncharged substances diffuse in plaque at about a half of their rates of diffusion in water. The diffusivity of both sodium acetate and sodium lactate in plaque was 0.31 (Table 10.1), a value intermediate between those for NaF and xenon. Thus the effect of electrostatic interaction on the diffusion of acetate and lactate is not as large as for fluoride, and this may be related to the higher charge density of the fluoride ion. Although the diffusivity in plaque of acetate and lactate is lower than for sucrose, the absolute rate of diffusion of acetate and lactate is faster than for sucrose by a factor of about 1.6. Since the hydrogen ion is more mobile than the sodium ion, acetic and lactic acids are expected to diffuse faster in plaque than the sodium salts, as happens in water. It is therefore concluded that these acids diffuse faster than sucrose in plaque.

It is convenient at this point to discuss in detail the results of two other workers who, during the period of research for this thesis, have performed experiments to measure rates of diffusion in plaque. Tatevossian (1979) studied the penetration of radiotracers into columns of plaque fluid and plaque residue which were separated from pooled, 24 h plaque by centrifugation at 5000 g for 15 min at 2°C. The sample was introduced into a 1-3 cm length of plastic tubing and a small amount of radiotracer applied at one end. After 5-17 h diffusion at 37°C, the sample was frozen, sliced into 1 mm segments and the amount of tracer in each segment determined by liquid scintillation counting. Assuming one dimensional diffusion

into an inert, homogeneous medium, the concentration of tracer c (cpm cm^{-3}), at any distance x (cm) and time(s), will be described by the following expression (Crank, 1956, p. 9-10; Redwood, Rall and Perl, 1974):-

$$c = m_0 (\pi Dt)^{-\frac{1}{2}} \exp\left(-\frac{x^2}{4Dt}\right) \quad \text{Equation 10.1}$$

where m_0 is the initial amount of tracer per cross-sectional area at the origin (cpm cm^{-2}). This equation was used to calculate diffusion coefficients of ^{14}C -labelled sucrose, inulin, starch, sodium bicarbonate, sodium acetate, sodium lactate and sodium butyrate in water, plaque fluid and plaque residue, and some of the results are summarised in Table 10.2.

The technique has low accuracy and precision. Diffusion coefficients for sucrose, bicarbonate, acetate, lactate and butyrate in water are between 24 and 42 per cent lower than previously reported values (see Appendix). Standard deviations exceed 30 per cent of the mean for nine of the 18 values of diffusion coefficient listed in Table 10.2, reaching 114 per cent for sodium bicarbonate in plaque fluid and 86 per cent for sodium acetate in water. There were some unexpected results. Starch was found to diffuse faster in plaque fluid than in water (both measured at 5°C) and sucrose diffused at the same rate in plaque fluid at 5 and 37°C , which conflicts with the Nernst and Einstein-Stokes formulae which predict faster diffusion at higher temperatures (see Appendix). Sucrose also diffused at the same rate in water and plaque fluid despite the higher viscosity of the plaque fluid. However, the diffusivity of sodium acetate and sodium lactate in plaque fluid was about 0.3, the same as reported in this thesis for diffusion in plaque.

Table 10.2 Summary of the diffusion coefficients and diffusivities in water, plaque fluid and plaque residue at 37°C, reported by Tatevossian (1979).

Diffusing substance	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$			Diffusivity	
	water	plaque fluid	plaque residue	plaque fluid	plaque residue
Sodium bicarbonate	12.0 \pm 2.2	2.8 \pm 3.2	0.94 \pm 0.15	0.23	0.08
Sodium acetate	10.6 \pm 9.1	3.2 \pm 0.8	1.3 \pm 0.2	0.31	0.12
Sodium lactate	10.1 \pm 2.5	3.4 \pm 1.8	1.4 \pm 0.2	0.33	0.14
Sodium butyrate	8.1 \pm 0.4	5.1 \pm 2.9	0.96 \pm 0.16	0.62	0.12
Sucrose	5.3 \pm 0.8	5.3 \pm 2.7	0.92 \pm 0.40	1.00	0.17
Inulin	3.3 \pm 1.0	2.9 \pm 0.9	0.41 \pm 0.14	0.88	0.12

\pm standard deviations

Diffusivity values for plaque residue were less variable than those for plaque fluid with a mean value of 0.125. As the packing density of the residue was increased by increasing the centrifugal force, the diffusivity of the residue decreased.

Some of these measurements may have been influenced by chemical and metabolic activity within the plaque fluid and residue. As the tracers diffuse they may be degraded, pass into bacterial cells, adhere to cell surfaces, attach to macromolecules or change their chemical form. Such reactions may be particularly important at the low concentrations of radiotracers used, so the penetration process would be complicated and perhaps not directly indicative of rates of diffusion. It is unfortunate that no measurements were made on plaque, although it might be speculated that such a study would yield values intermediate between those of plaque fluid and residue. In view of the report in this thesis that acetate and lactate diffuse faster than sucrose in plaque, it is of interest that Tatevossian reports that same relationship to apply for plaque residue, although the apparent diffusion coefficients are three-fold lower than the plaque values.

In a more recent study, Tatevossian and Newbrun (1980) reported rates of diffusion of some ionic species in plaque residue to be in the following order (fastest first):- NaCl , NaH_2PO_4 , Na_2HPO_4 , NaHCO_3 , Na_2SO_4 , CaCl_2 . Calcium penetrated more slowly than phosphate, which is contrary to the findings of Melsen et al. (1979) who used a similar technique to study the penetration of calcium and phosphate into plaque. The hypothesis of Melsen et al., that fixed negative charges in plaque matrix should retard diffusion of anions

and cations to different extents, therefore requires further investigation.

Dibdin (1979, and personal communication) has used a diaphragm cell technique to measure diffusion coefficients in pooled, 24 h plaque. Using a washer as a spacer, the sample was supported between two Millipore filters and formed a window between two fluid-filled chambers. Diffusion of radiotracers at 35°C from one chamber through the disc of plaque to the other chamber was then monitored over a 5 h time interval. After a short equilibration phase, the rate of flow of tracer will reach a steady value and the following expression will apply (Janz and Mayor, 1966; Jost, 1960, p. 11):-

$$D = \frac{1}{\beta t} \ln \left(\frac{C_1 - C_2}{C_3 - C_4} \right) \quad \text{Equation 10.2}$$

where D is the diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$),

t is the diffusion time (s),

C_1 and C_3 are the tracer concentrations in the first chamber at times 0 and t (cpm cm^{-3}),

C_2 and C_4 are the tracer concentrations in the second chamber at times 0 and t (cpm cm^{-3}), and

β (cm^{-2}) is a constant defined by:-

$$\beta = \frac{A}{l} \left(\frac{1}{V_1} + \frac{1}{V_2} \right) \quad \text{Equation 10.3}$$

where A is the area of the plaque window (cm^2),

l is the thickness of the plaque disc (cm),

and V_1 and V_2 are the volumes of each chamber (cm^3).

To prevent metabolic activity within the plaque, the samples were treated by heating to 60°C for 30 min and diffusion

coefficients were measured for some ^{14}C -labelled sugars and carboxylic acids. The results are summarised in Table 10.3 along with calculated values of diffusivity. The precision of the technique was good with standard deviations of less than 20 per cent of the mean. There was little variation in the diffusivities of these tracers, showing that plaque exhibits only a small degree of permselectivity, although the acids diffused slightly faster below their pK'_a 's. The diffusion coefficients were found to be dependent upon the packing density of the plaque but again show that acids diffuse faster than sugars.

The diffusion coefficients listed in Table 10.3 for acetate, lactate and sucrose are about half of the values reported in this thesis, but this may be a consequence of the different measuring techniques. Plaque may be considered to be composed of solid material with fluid-filled pores through which material can diffuse. The technique employed in this thesis directly measures the effective rates of diffusion in the pores. Only the sample thickness was substituted into Equation 3.4 to calculate diffusion coefficients. Thus the results from this thesis accurately describe the time required for penetration or clearance. To calculate the total amount of substance diffusing, the relative pore and solid volumes have to be known. By contrast, Dibdin measured the flux of material through the plaque sample i.e. the amount of tracer flowing per second through an area of 1 cm^2 , and calculated an apparent diffusion coefficient by substituting the total area of the plaque window into Equation 10.3. To calculate the rates of diffusion in the pores, only the cross-sectional area of the pores should be used. Taking this into consideration, the technique of Dibdin and of this thesis give

Table 10.3 Summary of the diffusion coefficients and diffusivities for plaque at 35°C reported by Dibdin (1979).

Diffusing substance	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^6$	Diffusivity
Acetate	2.5	0.20
Lactate	2.5	0.18
Propionate	2.5	0.19
Glucose	2.0	0.21
Fructose	2.0	0.21
Sucrose	1.3	0.18

consistent and complementary results.

The results of Tatevossian, Dibdin and of this thesis are in agreement that acids diffuse faster than sugars in plaque and that plaque does not act as a barrier to diffusion. This may seem to be in conflict with the observation that plaque pH in vivo may be lowered below resting values for more than 30 min following exposure to sucrose (Stephan curve). However, as discussed in Chapter 6, this could be a consequence of continued metabolism within the plaque rather than restricted diffusion of acids. Some of the sucrose is converted into intra- and extra-cellular polysaccharides which serve as a store of substrate for prolonged metabolic activity. These processes require further investigation and it would be of particular value to measure carbohydrate concentrations as well as pH in plaque following exposure to sucrose.

From the limited amount of evidence available from the three studies, it seems that dental plaque exhibits only a small degree of permselectivity. This effect may be of greater importance at low concentrations of diffusing substances where binding of ions may have a more pronounced effect on rates of penetration.

Most of the diffusion measurements have been made with plaque of 24 h accumulation. Further measurements should be made using older plaque because the structure and bacterial composition of plaque have been shown to change during a period of nine days accumulation (Ritz, 1967; 1969). The results reported in this thesis show no significant variations in diffusion coefficients in plaque from different subjects, but the number of subjects

investigated was relatively small, and should be increased. It would also be of value to study the diffusion of a wider range of substances in plaque. In an attempt to assess the diffusivity of macromolecules in plaque, Dibdin (1980) investigated the diffusion of ^{125}I -labelled gammaglobulin. However, the results were equivocal due to breakdown of the gammaglobulin.

It has been suggested (see Chapter 2) that extracellular polysaccharides and lipoteichoic acids might restrict rates of diffusion in plaque. This might lead to variations between plaques differing in composition, of diffusion rates and perhaps therefore of cariogenicity. With these suggestions in mind, the experiments of Chapters 7 and 8 were designed to assess the effect of glucans on diffusion. It was shown in Chapter 7 that glucans produced by Streptococcus sanguis 804 (NCTC 10904) do not retard the diffusion of NaF or sucrose but, to the contrary, these substances diffuse faster in glucan-rich than in glucan-free bacterial sediments. As different strains and species of bacteria produce different types of extracellular polysaccharides, it would be of value to extend the study to include other plaque bacteria and to study diffusion of acids as well as sugars. Chapter 8 contains further evidence that there is only a small variation in the rate of diffusion of NaF in plaque with different dietary histories and total carbohydrate contents. Incubation of plaque in sucrose-containing broth led to slightly faster rates of diffusion of NaF. This study should also be extended to include the diffusion of acids and sugars and analysis of the nature of the carbohydrate would be of value. The available evidence leads to the conclusion that

extracellular polysaccharides do not restrict rates of diffusion in plaque. However, extracellular polysaccharides could lead to an increase in the thickness of plaque accumulations in vivo. The time for diffusion is proportional to the plaque thickness to the second power (Equation 3.4). Thus the thickness of the plaque will be much more important with regard to diffusion times than the small variations in plaque diffusion coefficients arising from permselectivity or the presence of extracellular polysaccharides. Despite this, there is a paucity of data on the thickness of plaque layers in vivo.

In Chapter 9 it was shown that daily rinses with 0.2 per cent NaF solution caused a significant raising of the pH minimum of the Stephan curve. Such a rise may have a significant cariostatic effect by reducing the severity of acid attack on enamel, thus enhancing the more direct effects of fluoride in reducing enamel solubility and promoting remineralisation.

APPENDIX

ESTIMATION OF DIFFUSION COEFFICIENTS IN AQUEOUS SOLUTION AT 37°C

It is of value to compare rates of diffusion in dental plaque with the corresponding rates of diffusion in water. Many textbooks list values of diffusion coefficients but only for a limited range of solutes and temperatures and values of diffusion coefficients in water at 37°C for sodium fluoride, sucrose, sodium acetate and sodium lactate are not readily available. Fortunately, accurate estimates of these diffusion coefficients can be made using two well established relations.

The Nernst Equation relates the diffusion coefficient of a simple electrolyte to the mobilities of its constituent ions as follows (Robinson and Stokes, 1965, p. 288; Reid and Sherwood, 1966, p. 561):-

$$D^{\circ} = \frac{RT}{F^2} \frac{Z_+ + Z_-}{Z_+ Z_-} \frac{\lambda_+^{\circ} \lambda_-^{\circ}}{\lambda_+^{\circ} + \lambda_-^{\circ}} \quad \text{Equation A.1}$$

where D° is the diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) at infinite dilution,

R is the Gas Constant ($8.314 \text{ J } ^{\circ}\text{K}^{-1} \text{ mole}^{-1}$),

T is the temperature of the solution ($^{\circ}\text{K}$),

F is the Faraday Constant ($96500 \text{ coulombs equiv}^{-1}$),

Z_+ and Z_- are the valencies of the cation and anion

(sign ignored), and

λ_+° and λ_-° are the limiting ionic conductivities of the cation

and anion ($\text{cm}^2 \text{ohm}^{-1} \text{equiv}^{-1}$).

Thus, using the Nernst Equation, diffusion coefficients can be calculated for electrolytes, with an accuracy of about five per cent, from the conductivities of the constituent ions. Strictly, D° is the diffusion coefficient in an infinitely dilute solution, but in practice diffusion coefficients of simple electrolytes vary by only a small amount over a large range of concentration. As an example, the diffusion coefficient for KCl in water at 25°C is $1.99 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at infinite dilution, $1.83 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 0.3 M and $2.20 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 4 M (Longworth, 1972, p. 2-223) a variation of less than 20 per cent. Textbooks contain tables of ionic conductivities for an extensive range of ions, but unfortunately for a limited range of temperature.

However, diffusion coefficients can be extrapolated from one temperature to another with an accuracy of five per cent or less using the Einstein-Stokes formula (Robinson and Stokes, 1965, p.44):-

$$D = \frac{kT}{6\pi\mu r} \quad \text{Equation A.2}$$

where D is the diffusion coefficient ($\text{cm}^2 \text{ s}^{-1}$),

k is the Boltzmann Constant ($1.381 \times 10^{-23} \text{ J } ^\circ\text{K}^{-1}$),

T is the temperature of the liquid ($^\circ\text{K}$),

μ is the viscosity of the liquid (cp), and

r is the radius of the diffusing ion or molecule (m).

It can usually be assumed that r does not change with temperature, so that:-

$$D = \text{constant} \frac{T}{\mu}$$

and therefore
$$\frac{D_1 \mu_1}{T_1} = \frac{D_2 \mu_2}{T_2} \quad \text{Equation A.3}$$

where subscripts 1 and 2 refer to the different temperatures.

Equation A.3 can be rearranged to the following form:-

$$D_2 = D_1 \frac{\mu_1}{\mu_2} \frac{T_2}{T_1} \quad \text{Equation A.4}$$

Thus, using the Nernst and Einstein-Stokes Equations, diffusion coefficients in aqueous solution at 37°C can be calculated from values of ionic conductivity. The following three examples illustrate the use of this method to calculate diffusion coefficients for NaF, sucrose and acetic acid.

Sodium fluoride The limiting ionic conductivities for Na⁺ and F⁻ in aqueous solution at 25°C are 50.1 and 55.4 cm² ohm⁻¹ equiv⁻¹ respectively (Robinson and Stokes, 1965, p. 463). Using the Nernst Equation:-

$$\begin{aligned} D_{25}^{\circ} &= \frac{RT}{F^2} \frac{Z_+ + Z_-}{Z_+ Z_-} \frac{\lambda_+^{\circ} \lambda_-^{\circ}}{\lambda_+^{\circ} + \lambda_-^{\circ}} \\ &= \frac{(8.314)(298)}{(96500)^2} \frac{1 + 1}{(1)(1)} \frac{(50.1)(55.4)}{50.1 + 55.4} \\ &= \underline{1.40 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}} \end{aligned}$$

The viscosity of water at 25 and 37°C is 0.890 and 0.692 cp, respectively (Robinson and Stokes, 1965, p. 465) so from the Einstein-Stokes Equation:-

$$\begin{aligned}
 D_{37}^{\circ} &= D_{25}^{\circ} \frac{\mu_{25}}{\mu_{37}} \frac{T_{37}}{T_{25}} \\
 &= 1.40 \times 10^{-5} \frac{0.890}{0.692} \frac{310}{298} \\
 &= \underline{1.87 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}}
 \end{aligned}$$

Sucrose The diffusion coefficient of sucrose in aqueous solution at 25°C is $5.233 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ at infinite dilution and $4.846 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ at a concentration of about 150 mM (Longworth, 1972, p. 2-224). Using the Einstein-Stokes Equation:-

$$\begin{aligned}
 D_{37}^{\circ} &= D_{25}^{\circ} \frac{\mu_{25}}{\mu_{37}} \frac{T_{37}}{T_{25}} \\
 &= 5.233 \times 10^{-6} \frac{0.890}{0.692} \frac{310}{298} \\
 &= \underline{7.00 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}}
 \end{aligned}$$

At very high sucrose concentrations (above 1 M), the rate of diffusion of sucrose falls rapidly with increasing concentration due to a large increase in the viscosity of the solution. However, these concentrations will seldom be encountered in the mouth e.g. the concentration of sucrose in a cup of tea containing three teaspoonfuls of sugar is about 300 mM and this will be diluted by mixing with saliva in the mouth.

Acetic acid The diffusion coefficient of acetic acid in aqueous solution at 20°C is $1.19 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at infinite dilution (Reid and Sherwood, 1966, p. 555). The viscosity of water at 20°C is 1.000 (Tennent, 1971, p. 62) so from the Einstein-Stokes Equation:-

$$\begin{aligned}
 D_{37}^{\circ} &= D_{20}^{\circ} \frac{\mu_{20}}{\mu_{37}} \frac{T_{37}}{T_{20}} \\
 &= 1.19 \times 10^{-5} \frac{1.000}{0.692} \frac{310}{293} \\
 &= \underline{1.82 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}}
 \end{aligned}$$

Diffusion coefficients of various electrolytes in aqueous solution at 37°C, referred to throughout this thesis, were calculated in the same way. For the electrolytes listed in Table A.1, diffusion coefficients at 25°C have been published (Longworth, 1972, p. 2-222) and these values were extrapolated to 37°C using the Einstein-Stokes Equation. For the sodium salts listed in Table A.2, limiting ionic conductivity values at 25°C have been published (Dean, 1979, pp. 6.38-6.43). The Nernst Equation was used to calculate diffusion coefficients at 25°C and these were extrapolated to 37°C using the Einstein-Stokes Equation.

Table A.1 Diffusion coefficients of some electrolytes at infinite dilution in aqueous solution at 25°C (Longsworth, 1972, p. 2-222) and values calculated for 37°C using the Einstein-Stokes Equation.

Electrolyte	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$) $\times 10^5$	
	25°C	37°C
NaCl	1.61	2.15
KCl	1.99	2.66
KI	2.00	2.68
HCl	3.34	4.47
NH_4Cl	1.84	2.46
CaCl_2	1.34	1.79
Na_2SO_4	1.23	1.65

Table A.2 Limiting ionic conductivities for some anions in aqueous solution at 25°C (Dean, 1979, pp. 6.38-6.43), diffusion coefficients for the sodium salts at 25°C calculated using the Nernst Equation and then extrapolated to 37°C using the Einstein-Stokes Equation. The limiting ionic conductivity of sodium at 25°C is 50.1 cm² ohm⁻¹ equiv⁻¹ (Dean, 1979).

Anion	Limiting ionic conductivity of anion at 25°C (cm ² ohm ⁻¹ equiv ⁻¹)	Diffusion coefficient of sodium salt (cm ² s ⁻¹) x 10 ⁵	
		25°C	37°C
formate	54.6	1.39	1.86
acetate	40.9	1.20	1.61
lactate	38.8	1.16	1.55
propionate	35.8	1.11	1.49
succinate (2-)	58.8	1.08	1.44
butyrate	32.6	1.05	1.40
citrate (3-)	70.2	1.04	1.39
bicarbonate	44.5	1.25	1.67
H ₂ PO ₄	33	1.06	1.42
HPO ₄ (2-)	57	1.06	1.42

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