The Teeth and Their Environment

Physical, Chemical and Biochemical Influences

Editor

R.M. Duckworth

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The Teeth and Their Environment

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Physical, Chemical and Biochemical Influences

Volume Editor

Ralph M. Duckworth   Wirral

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Preface

Tooth enamel is the hardest tissue in the human body. Many aspects of the oral environment have evolved to aid the stability of the teeth in order to preserve their natural function throughout life. Despite this underlying strength and supporting environment, a variety of factors conspire to ensure that almost everyone suffers damage to their teeth at some time. The aim of this monograph is to provide an overview of intrinsic and extrinsic factors that influence the state of the teeth, with emphasis on those factors that have received either comparatively little attention in the literature or justified an updated review in the opinion of the authors. Thus, the scope of this monograph is caries, calculus, tooth wear and erosion, and the roles of pellicle, saliva and plaque in inducing and/or moderating these conditions. A contribution on tooth structure underpins much of what is discussed elsewhere. In the interest of space and to maintain focus, the content is restricted to physical, chemical and biochemical aspects.

Each chapter is written by experts in their respective fields. In describing their chosen topics, authors have sought wherever possible to illustrate major points with examples from their own work.

Chapter 1 provides an update on salivary and plaque factors in the aetiology and control of caries and calculus, that builds on earlier reviews. Particular emphasis is given to the inverse association between caries and calculus often observed in clinical studies. Whilst this relationship is intuitively reasonable from the perspective of the chemistry involved, few researchers have been able to demonstrate links to putative common causative factors. The present authors first establish that the inverse association is based on sound clinical data,

utilizing the database of the large-scale toothpaste clinical trials sponsored by Unilever Oral Care Research over decades. The authors then seek to identify significant causative factors from the extensive published literature on saliva, plaque and their constituents.

In *Chapter 2* the structure and function of the acquired enamel pellicle are discussed. Recent work, aided by advances in analytical methodologies, has increased our understanding of pellicle formation and maturation, and how pellicle composition relates to saliva. The authors emphasize the important role played by pellicle in plaque attachment, tooth protection and stain formation. The authors also describe research on the potential effects that dentifrices can have on pellicle.

Continuing the theme of saliva function, *Chapter 3* describes environmental factors that affect the mineralization of hard tissue. The author describes in vitro investigations by himself and others on the relative roles of saliva and plaque fluid, and how the presence or absence of a biofilm affects the efficacy of anticaries agents. Other work focuses on the effects of lesion composition and of lesion proximity to dentine on subsequent enamel de- and remineralization behaviour. The author also discusses the importance of how the characteristics of pre-formed artificial lesions can influence subsequent mineralization behaviour in both in vitro and in situ studies.

A topic of increasing interest is tooth wear. *Chapter 4* describes recent research on the influence of dental product use, diet and other natural factors, and the inter-relationship between abrasive wear and chemical erosion. After describing the various physical and chemical mechanisms implicated in tooth wear, the author discusses the laboratory, in situ, and clinical approaches used to investigate the condition, illustrated with selected examples.

Chapter 5 concerns the mechanical properties of tooth mineral, with particular emphasis on the use of nanoscale hardness measurements to elucidate the variations across the tooth surface and how they may be associated with tooth function. The influence of environmental factors, such as those described in Chapter 4, are also discussed. In addition, the authors present very recent studies, employing a variety of state-of-the-art techniques, on pellicle-coated enamel and on the early carious lesion, which complements the work described in Chapters 2 and 4, respectively.

The important role of plaque as a reservoir for active agents, such as fluoride from dental products and calcium and phosphate from saliva, is reviewed in *Chapter 6*. Recent findings on the dependence of the concentration and retention of these agents on plaque location are included. The relationship between plaque fluid chemistry and tooth mineral is the key to caries formation and control. This interdependence is also discussed in Chapter 3. Here both the thermodynamic approach of Margolis and coworkers and the kinetic approach of

Dawes, Dibdin and coworkers are contrasted. Of special interest, the authors present recent plaque data from subject groups in China, who have experienced different life histories to those from communities normally investigated.

Many of the studies reported in this monograph were either conducted by scientists of Unilever Oral Care Research at Unilever Research & Development Port Sunlight UK or were sponsored by Unilever. On behalf of the various contributors to this monograph, I thank Unilever Oral Care Research for permission to publish that work and for financial support to Karger AG, Basel to facilitate publication.

<div align="right">Ralph M. Duckworth, Monograph Editor, March 2005</div>

Chapter 1

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On the Relationship between Calculus and Caries

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1.1 Introduction

From a mechanistic viewpoint it is reasonable to anticipate an inverse clinical relationship between calculus and caries. Calculus formation is essentially a *mineralisation* process. The development of a caries lesion is the result of the net *demineralisation* of tooth enamel by plaque acid. These processes both involve crystalline calcium phosphate phases in contact with liquid, saliva and/or plaque fluid, containing their constituent ions. The oral environment also contains other salivary constituents and bacteria, which either inhibit or promote crystal growth or dissolution.

An inverse relationship would mean that the absence of calculus could be a useful predictor of caries. Historically, however, any calculus–caries relationship has often been obscured by other factors. Firstly, the prevalence of both calculus and caries increases with increasing age [1, 2] and, second, both conditions are expected to correlate positively with poor oral hygiene [3–5]. These trends could be the reason why Schroeder [1] found no consistent relationship between clinical observations of calculus and caries experience in the first major review of the topic.

The main purpose of this chapter is to review data from several clinical trials sponsored by Unilever, in which both caries and supra-gingival calculus were assessed concomitantly, in order to assess the strength of any empirical relationship between the two conditions. These data are presented in the following section 1.2, together with the results of other relevant published studies. To provide background information against which to judge the findings derived from the clinical data, calculus formation and caries are then briefly described

Contents

Table 1. Calculus scoring systems employed in Unilever-sponsored clinical trials

Type	Scoring system	Where used
I	0 = no calculus 1 = calculus on anteriors only 2 = calculus on molars	Lanarkshire [8] and Somerset [9]
II	1 = no calculus 2 = sub-gingival calculus 3 = supra-gingival calculus 4 = sub- and supra-gingival calculus	Bristol [10], Berkshire [11], Munster [12], Villefranche [13] and Isle of Lewis [14]

For the current data analysis, the presence of calculus was denoted by scores of 1 or 2 for the type I index and 3 or 4 for the type II index. The exception is the Villefranche trial where no 4s and very few 3s were recorded, so that 2 or 3 have been used.

in section 1.3. The possible roles of saliva and plaque are introduced in section 1.4, followed by a review of studies of relationships between various salivary and plaque factors and calculus or caries in section 1.5.

1.2 Clinical Results

The number of caries clinical trials that have been published is over one hundred [6, 7]. However, the majority do not appear to have included an assessment of calculus.

Of those studies in which both conditions have been monitored, it will become clear below that the anticipated inverse relationship between calculus and caries is much more evident in the currently analysed, Unilever-sponsored, clinical trials than in many others. A major reason for this is that the former studies were mostly restricted to a narrow age range of subjects.

1.2.1 Unilever-sponsored studies
There have been a number of Unilever-sponsored 3-year caries clinical trials during which calculus prevalence was also recorded. Table 1 summarises the variations in the scoring system employed. For the purpose of the present analysis, scores have been combined to produce two subject groups: those with calculus and those without calculus. Mean baseline caries and calculus prevalence data from six trials are listed in table 2, whilst corresponding 3-year caries increment data and calculus prevalence at the end of each trial are listed in table 3.

Table 2. Relationship between caries and calculus prevalence at baseline [subjects in all studies aged 11–13 years at baseline]

Trial	Date	N**	Calculus-free subjects+		Calculus-prone subjects+		Caries difference* (%)	p value++
			N	Mean DMFS	N	Mean DMFS		
Bristol [10]	1970/73	742	620	8.85	122	7.11	19.7	0.01
Berkshire [11]	1974/77	1203	819	9.22	384	8.09	12.3	0.01
Munster [12]	1976/79	1305	903	12.31	402	10.48	14.9	0.0001
Somerset [9]	1976/79	1319	728	6.62	591	5.60	15.4	0.01
Villefranche [13]	1979/82	1061	898	5.62	163	5.53	1.6	ns
Lanarkshire [8]	1983/86	2316	1538	11.08	778	8.40	24.2	0.0001

+calculus assessed as either present or absent.
++statistical significance of caries difference.
*[DMFS (calculus-free) − DMFS (calculus-prone)]/DMFS (calculus-free).
**number of subjects who completed trial.

The baseline data of table 2 show that caries prevalence is significantly lower in calculus-prone subjects than in calculus-free subjects in 5 of the 6 studies (average difference = 16%). The caries difference varies from one study to another, possibly because of differences in the clinicians' interpretation of the scoring system, but overall the inverse relationship between calculus and caries is clear.

The inverse relationship is again manifested in the increment data of table 3a. Children classified as calculus-formers at the start of a trial produced over 20% fewer caries lesions on average during the 3 years than their initially calculus-free counterparts. The baseline classification for calculus proneness also proved to be a good predictor of calculus status at the end of a trial.

It is perhaps not surprising that a similar relationship exists between baseline calculus status and both mean baseline caries prevalence and corresponding mean 3-year caries increment. This is because a direct relationship between baseline caries prevalence and subsequent caries incidence has long been established. For example, we demonstrate elsewhere [17] that such a relationship exists for the Lanarkshire clinical trial data. Moreover, when the same data are separated into baseline calculus-prone and calculus-free groups, the former group has a consistently lower caries increment over the entire range of baseline caries prevalence.

A comparison of the caries prevalence data of table 2 and the corresponding caries increment data of table 3b shows that the percentage difference in

Table 3a. Relationship between calculus prevalence and caries incidence at trial end

SMFP concentration (ppm F) Trial	Baseline calculus status	0		1000		1500		2500		% caries reduction* (1000 v. 0)
		% calculus-formers at end	Mean DMFS increment	% calculus-formers at end	Mean DMFS increment	% calculus-formers at end	Mean DMFS increment	% calculus-formers at end	Mean DMFS increment	
Bristol	free	13	8.13	20	6.44					20.8
	former	75	6.39	75	4.64					27.4
Berkshire	free	23	6.49	28	4.53					30.2
	former	61	4.41	74	3.29					25.4
Munster	free	20	15.12	21	12.61	22	11.22			16.6
	former	72	12.78	69	9.41	71	9.24			26.4
Somerset	free			24	5.62					
	former			72	5.27					
Villefranche	free	10	7.67			12	5.85			23.7
	former	61	6.72			68	4.66			30.6
Lanarkshire+	free			11	7.59	11	7.06	6	6.76	
	former			40	5.10	51	5.03	51	4.92	

+only users of non-zinc citrate containing pastes (N = 1172).
*Villefranche trial: 1500 v. 0.

Table 3b. Percentage reduction in caries increment between calculus-formers and calculus-free subjects

SMFP concentration (ppm F) Trial	0	1000	1500	2500	Weighted average
Bristol	21.4	28.0			24.7
Berkshire	32.0	27.4			28.9
Munster	15.5	25.4	17.6		19.5
Somerset+		6.2			6.2
Villefranche	12.4		20.3		16.4
Lanarkshire		32.8	28.8	27.2	30.1
weighted average	19.6	19.7	22.3	27.2	20.5

+The relatively low value for this trial could be because the diagnostic boundary between calculus-free and calculus-prone subjects was less sharp compared to other trials. The proportion of subjects diagnosed as calculus-formers was highest in this trial (see table 2). On omitting the value for Somerset, the overall weighted average (bottom right-hand number) increases from 20.5% to 23.9%.

Table 4. Relationship between caries incidence and calculus prevalence at the end of the Isle of Lewis study

Toothpaste used during trial	Calculus-free subjects		Calculus-prone subjects#	
	N	Mean DMFS*	N	Mean DMFS*
Control	59	15.80	51	14.04
2500 ppm F	47	13.87	71	10.07
% caries reduction		12.2		28.3

subjects with calculus status coded 3 or 4.
* 6-year caries increments for teeth unerupted at baseline.

caries between the calculus-prone and calculus-free groups appears to have increased on average from the beginning to the end of the 3-year trial. This suggests a possible cumulative effect with age.

Table 3b also highlights the fact that the inverse calculus–caries relationship is largely independent of fluoride experience. Four fluoride concentrations were tested over the 6 trials: 0, 1000, 1500 and 2500 ppm F as sodium monofluorophosphate (SMFP or Na_2FPO_3) in alumina- or insoluble sodium metaphosphate-based dentifrices. Average differences in caries increments between calculus-prone and calculus-free subjects for each fluoride level were all in the range 20–27%.

Of great importance, table 3 provides a good indication of the clinical, as opposed to statistical, significance of the caries–calculus relationship for the age group tested – teenagers. The difference in caries increment between calculus-formers and non-formers (20.5%, table 3b) is of similar magnitude to the average caries reduction found for the 1000 ppm F dentifrice compared to the non-fluoridated control dentifrice in the present trials (24%, calculated from final column of table 3a) and in published trials generally, ca. 21% [6, 7].

A further data set, which also confirms the caries–calculus association, is shown in table 4. These results are from a study of children, initially aged 8 years, conducted on the Isle of Lewis [14] that comprised a 3-year clinical trial followed by another clinical examination 3 years later. The 6-year caries increments are lower for children who exhibited calculus at some time during the study than for those who were always calculus-free. This tendency was found both for children who had used a 2500-ppm F dentifrice during the clinical trial and those who had used a non-fluoridated control dentifrice.

There is also a tendency in the trials summarised in table 3, and in the study analysed in table 4, for the presence of fluoride to be more beneficial

against caries in calculus-formers than in subjects who were calculus-free. An explanation for such behaviour is that calculus provides a reservoir for orally retained fluoride. Such fluoride would have to be surface-bound to calcium phosphate, as the fluoride in fluorhydroxyapatite, suggested to be the most likely form of F in calculus [15], would not be labile.

The data in table 3a also suggest that the presence of fluoride in the test toothpastes had little or no effect on the proportion of calculus-formers at the end of the trials. This finding is consistent with the observation of Jin and Yip in their recent review of calculus [16]. They implied that the cause was a balance of fluoride effects on potential factors that could promote or inhibit calculus formation.

The above studies all involved children, whereas calculus is more prevalent in adults. In a very recent paper [17], we discuss a large-scale calculus clinical study [18] that involved a wide age range of subjects (20–65 years). For most sub-groups of narrow age range, Volpe-Manhold calculus increment scores were significantly inversely correlated with caries prevalence on an individual basis, even though only calculus-formers took part. A notable feature of this study is that both caries and calculus increased with increasing age of subjects, as expected. This behaviour masked the relationship between the two parameters when the data set was analysed as a whole. This could well be the reason for the apparently inconsistent findings of other authors (see 1.2.2).

We conclude that the above Unilever-sponsored studies demonstrate unequivocally an inverse relationship between calculus and caries for subjects of similar age. Furthermore, the magnitude of the difference in caries experience between calculus-formers and calculus-free schoolchildren is clinically significant. Baseline calculus status would therefore appear to be a useful stratifying factor during subject selection for caries clinical trials.

Indeed, calculus status has been successfully employed in more recent trials sponsored by Unilever [19–21]. One example is the 3-year caries clinical trial of Stephen et al. [19], which involved 4,294 children at the outset and compared the efficacy of 6 different toothpastes. The factors used for stratification were: clinician (of which there were two), gender (male/female), presence of supragingival calculus on lower incisors (yes/no) and caries status at baseline (four categories). The general linear model used for data analysis included all of these factors together with: active type (NaF or SMFP), fluoride concentration (1000 or 1500 ppm), plus all two-level interactions. After excluding non-significant terms, the model reduced to: DMFS increment = linear function of (baseline caries status, baseline calculus, interaction between baseline calculus and baseline caries, active type). The result of this analysis revealed mean DMFS increment to have highly significant associations with baseline caries status ($p < 0.0001$), baseline calculus ($p < 0.0001$) and the interaction between these two parameters ($p < 0.0003$).

The above authors noted that both subsets of subjects, with and without calculus, showed the expected monotonic increase in DMFS increment with caries status category. However, the difference between subjects with calculus and those without calculus at baseline increased progressively with caries status. In a later clinical trial, of simpler design but in which a similar stratification procedure was adopted, mean 3-year DMFS increment again had a highly significant association with baseline calculus [20]. These findings confirm that the presence of supragingival calculus is a good indicator of caries susceptibility.

1.2.2 Other Published Studies

There are few reports in the literature on the correlation between caries and supra-gingival calculus. In his major review of calculus in 1969, Schroeder [1] cited four studies, of which only two showed the anticipated negative correlation that predominates in the studies discussed in section 1.2.1. The other studies showed no relationship. Furthermore, Schroeder pointed out that in one of the two examples of a negative correlation, the relationship was misleading. The subject population were Eskimos in whom caries prevalence was markedly lower for ages over about 18 years old, the opposite of the usual trend. This finding was thought to be caused by a change in diet of the younger age groups. The only convincing, and statistically significant, negative correlation between supra-gingival calculus and caries up to that time, therefore, was by Marthaler and Schroeder [22]. They probably achieved this result because the age range of their subjects was narrow, 8–15, and the study population was large, 4300.

Few papers that mention a possible association between calculus and caries have been reported since that time. In the most focused of these, Manji et al. [23] presented data from an oral health study involving 1131 Kenyans aged 15–65 years. By dividing the subjects into narrow age bands, these authors were able to demonstrate an inverse, but weak, association between calculus and caries. They concluded that the correlation was not strong enough to be of clinical significance. Of five further relevant studies [24–29], an inverse association between calculus and caries was reported in three [24, 26, 27]. In each case, the correlations were weaker than those reported in section 1.2.1.

Crossner and Holm [24] observed a significant inverse correlation between supra-gingival calculus and caries in the primary teeth of 149 eight-year-old children, but not between calculus and caries in the permanent teeth. These authors commented that their careful clinical diagnosis of calculus (as indicated by the higher prevalence of calculus found than in an earlier study) was the probable explanation for their study confirming the inverse relationship in such a young age group. The lack of a significant correlation in the permanent dentition would have been due to the relatively low number of such teeth erupted in the children.

Cahen et al. [25] found that the prevalence of both calculus and caries increased with age in an oral health study of 1993 young adults but did not report any correlation between the two parameters. In an earlier study [26] of 2000 children, the same research group estimated calculus to be fourth in the order of factors influencing caries prevalence after subject age, social group of father, and sex. The correlation between caries and calculus was negative, in agreement with expectation.

A weak, but statistically significant, negative correlation between calculus and caries incidence was found by Berkey et al. [27] in a 10-year longitudinal study of caries lesion progression in 602 adults. Calculus score correlated much better with plaque, gingivitis and pocket depth, whilst caries score correlated best with plaque, baseline caries and subject age. Overall, this study highlights another probable masking factor of any calculus–caries relationship, general oral hygiene.

Geary et al. [29] reported qualitatively similar findings in their multivariate analyses of four caries clinical trials, in which plaque, gingivitis and calculus were also recorded: caries incidence correlated best with baseline caries prevalence and calculus with gingivitis.

De Paola et al. [28], in a study of the clinical profiles of 273 adults with and without root surface caries, found that those without root caries had less coronal caries and less calculus, as well as more teeth, less recession, less debris, less gingivitis and more abrasion, than subjects with root caries. They attributed 'most, if not all, of the differences' to one underlying factor, oral hygiene.

Recent calculus reviews [16, 30, 31] have not mentioned the possible relationship between caries and calculus. White [31] noted correlations between supra-gingival calculus and factors such as plaque and oral hygiene but not caries, though his review is focussed more on potential links to gingivitis. However, two papers in this time period do discuss the relationship.

Pattanaporn and Navia [32] reported a study of calculus, caries and gingivitis in a young Thai population with high calculus prevalence. They found no relationship between calculus and caries, which may have been because their 'no calculus' group was not actually calculus-free and/or because caries prevalence was low, 42% of subjects being caries-free.

In a study of calculus inhibition by beta-blocker drugs, Breuer et al. [33] found that a group of adults who regularly used such medication had significantly less calculus than a control group of similar age range who took no medication. Of interest, the prevalence of root caries in the former group was higher than in the latter group, consistent with an inverse relationship between the two conditions. The authors were unable to explain the role of the beta-blockers in calculus inhibition from amongst several possibilities.

1.3 Overview of Calculus and Caries Aetiology

1.3.1 Calculus Composition and Formation

Dental calculus, or tartar, occurs as a calcified deposit around the teeth. When the deposit occurs above the gum margin it is known as supra-gingival calculus, while that below the gum margin is known as sub-gingival calculus. Calculus is variable in composition but always contains 40–80% of inorganic mineral with the proportion increasing with the age of the deposit [34]. This mineral consists mainly of calcium phosphates: hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$; octacalcium phosphate (OCP), $Ca_8(HPO_4)_2(PO_4)_4$; whitlock-ite, $(Ca,Mg)_3(PO_4)_2$; and dicalcium phosphate dihydrate (DCPD or brushite), $CaHPO_4.2H_2O$ [35].

Analyses suggest that plaque fluid is supersaturated with respect to the above phases [36] whilst saliva may not be supersaturated with respect to the most soluble phase, DCPD [37, 38]. Of prime importance, the degree of super-saturation tends to increase with increasing pH (fig. 1). A key determinant of calculus formation may be a small pH rise, because of either loss of carbon dioxide, a natural buffer from saliva, or bacterial metabolic activity [40, 41]. A corollary to this is the observation in animals that a high sugar diet, which would have led to a lowering of plaque pH (see below), resulted in low calculus and high caries [42]. Other essential factors for initiating precipitation are the presence of suitable nucleation sites and of only an insignificant level of crystal growth inhibitors [43]. Plaque bacteria have been implicated in crystal nuclea-tion [44, 45] and also plaque contains a number of enzymes capable of degrad-ing inhibitors that occur naturally in saliva [46, 47]. These factors are discussed more fully in section 1.4. Plaque is also believed to be a precursor of calculus formation because plaque bacteria are often seen as the foci of calcification in X-ray [48] and electron microscope studies [49].

1.3.2 Carious Lesion Formation and Repair

In contrast to calculus, dental enamel contains over 96% w/w inorganic mineral [50]. The main constituent is a single calcium phosphate phase, HAP, the structure of which contains minor impurities such as magnesium, sodium, carbonate and chloride [50]. Dental caries is a disease of bacterial origin. Certain plaque bacteria can ferment sugars and other carbohydrates from the diet to produce lactic acid and other short chain organic acids [51]. If the con-centration of acid depresses the pH adjacent to the tooth surface below about pH 5.5, then the enamel dissolves.

In the absence of therapeutic agents, tooth minerals are continually being exchanged between saliva and tooth enamel. Under the above acidic conditions enamel dissolves, which leads to the formation of a carious lesion. However,
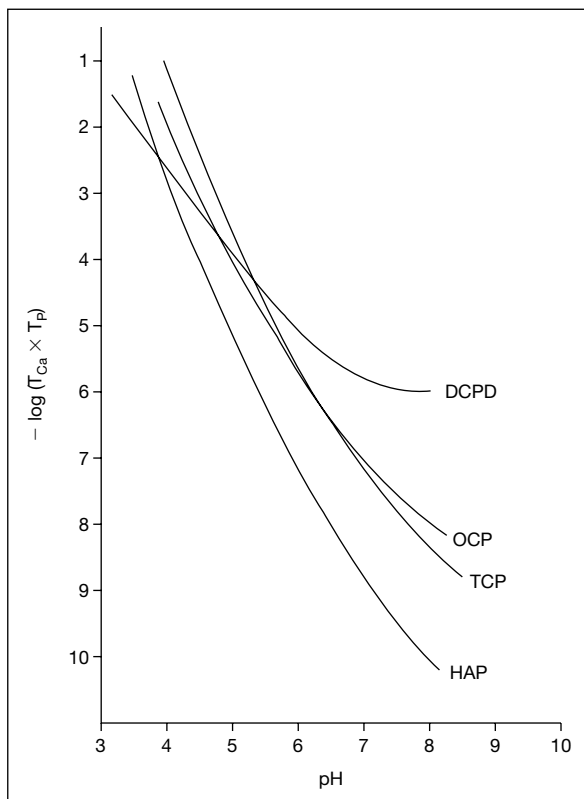
Fig. 1. Stability isotherms of calcium phosphates. Plots are of $-\log(T_{Ca} \times T_P)$ against pH for saturated solutions at an ionic strength of 0.01 mol/l, reprinted from ref. [39] with permission from Elsevier. T_{Ca} and T_P are total solution concentrations of calcium and phosphate respectively; TCP is tricalcium phosphate, $Ca_3(PO_4)_2$, which is stoichiometrically similar to whitlockite; for other abbreviations see text. For saliva, the value of $-\log(T_{Ca} \times T_P)$ is about 5.8 [37], whilst the corresponding value for plaque fluid is about 5.0 [36], at average oral pH values close to 7.

under conditions closer to pH 7, enamel tends to re-acquire minerals and incipient lesions are repaired. This cycle of alternating pH can be viewed as an oscillation about the HAP solubility boundary in fig. 1 (for the appropriate ion product), and is discussed in more detail elsewhere [52, 53]. For caries-susceptible people, the above balance between enamel dissolution and repair favours lesion formation, whereas for caries-free people the balance favours lesion repair.

1.4 Possible Roles of Saliva and Plaque

It is evident from the above that saliva and plaque normally play key roles in the formation of calculus and caries. A possible, but perhaps artificial, distinction between the two conditions is that calculus requires saliva, to supply the necessary minerals for crystal growth, whilst caries requires plaque, to provide the necessary acid for crystal dissolution. Saliva contains inhibitors of both processes.

1.4.1 Saliva

The calculus reviews cited earlier [1, 16, 30, 31] discuss the role of saliva and its constituents in the context of mineral formation and dissolution in plaque. Other authors have reviewed saliva with respect primarily to dental caries [e.g. 54, 55], caries models [56, 57] and the acquired pellicle [58].

Normally, saliva is continuously flowing through the mouth and, hence, the various active constituents are constantly being renewed. The importance of saliva in calculus formation is highlighted by the localised distribution of supra-gingival calcified deposits close to the sites of the main salivary glands, on the lingual surfaces of the lower teeth and on the buccal surfaces of the upper molars [59, 60]. The need for the continuous supply of saliva to protect the teeth against caries is demonstrated by the phenomenon of salivary gland hypofunction. People who suffer from this drug- or disease-induced reduction in normal saliva flow are subject to high levels of caries [61]. Saliva flow *per se* is often cited as the single most important factor in determining caries risk [54, 55]. However, on an individual basis, the salivary output of healthy subjects can differ dramatically [62] and studies that demonstrate a clear negative correlation between salivary flow rate and caries activity are few [55, 62, 63].

Many components of saliva may affect mineralisation processes, either directly or indirectly. The compositions of the secretions from the different salivary glands are different. Moreover, salivary composition differs with flow rate, which, in turn, depends on the time of day, type of stimulus, etc. [50, 64]. Salivary composition may also change with subject age, although evidence is lacking. The discussion here is restricted to the properties of individual components. However, clinical studies investigating possible relationships between salivary factors and calculus and/or caries need to take account of the above variations in composition. Indeed a recent paper suggested that such variations rendered this type of investigation almost meaningless [65]. However, the authors based this conclusion on data derived from repeat saliva samples collected in the morning, a time when flow rate and other salivary parameters are subject to marked change because of circadian rhythms [64].

1.4.1.1 Inorganic Factors

Saliva is supersaturated with respect to all the calcium phosphate phases identified in calculus and enamel except possibly DCPD. Salivary pH controls the degree of supersaturation (fig. 1), and is generally in the range pH 6.5–pH 7. The pH buffering capacity of saliva, determined mainly by the concentrations of bicarbonate and phosphate [50], limits the extent and duration of the pH drop that results from plaque acid formation. Potentially important local differences in pH can occur because saliva flow is restricted in certain regions of the mouth, e.g. interproximally, and is rapid in others [60]. Saliva also contains components that tend to inhibit crystal growth naturally, of which inorganic pyrophosphate is one example [66, 67].

1.4.1.2 Organic Factors

A variety of organic salivary components have the potential to influence calculus and caries. These include:

— non-enzymatic functional proteins, e.g. glycoproteins (or mucins), proline-rich proteins (PRPs), statherin, cysteine-containing phospho-proteins (CCPs), histidine-rich proteins and immunoglobulin A (IgA).
— enzymes, e.g. acid phosphatase, lysozyme, peroxidase and amylase.
— Lipids, e.g. cholesterol, fatty acids and glycolipids.
— Urea, sialin.

The most important components are probably the proteins, and the possible roles of these molecules in caries, in particular, have been studied extensively. An obvious inhibitory function of the salivary proteins is the formation of an adsorbed layer on the teeth, the acquired pellicle. For more details on the composition and function of pellicle the reader is referred to chapter 2 and ref. 58.

Both salivary urea and sialin, a tetrapeptide containing arginine, are metabolised by certain oral bacteria to produce ammonia which may contribute to the pH buffering of plaque [41, 68–70].

1.4.2 Plaque

Plaque is an essential prerequisite for dental caries to occur, since it is plaque bacteria that produce the acid which dissolves the enamel. However, plaques are of varying cariogenicity, which depends to a large extent on their bacterial composition. In addition, plaque thickness should affect cariogenicity because thickness determines the extent of any acid challenge to the teeth [71, 72]. Increasing plaque thickness would also be expected to minimise the potentially protective influence of dissolution inhibitors in saliva.

In addition to shielding the tooth surface from fresh supplies of dissolution inhibitors, plaque bacteria are also a source of enzymes that can cause their degradation; for example, alkaline phosphatase and protease [46, 47].

The fact that plaque is a region in the mouth relatively free from salivary inhibitors is probably one reason why calcification, leading to calculus formation, is associated with plaque. A second reason is the presence of certain bacteria that calcify, e.g. *Corynebacterium* (formerly Bacterionoma) *matruchotii* and *S. sanguis* I/II (more recently *S. gordonii*). The former bacterium contains cell wall components that can nucleate crystal growth [44, 45, 73], whilst the latter bacterium can metabolise basic amino acids, [74] and thereby, locally raise pH, which favours calcium phosphate precipitation.

1.5 Saliva and Plaque Factors Associated with Clinical Calculus and/or Caries

The clinical evidence presented in section 1.2 unequivocally supports an inverse association between calculus and caries experience for subjects of similar age. There are many possible reasons for this finding. In section 1.4 a variety of saliva and plaque factors were discussed which have been shown to influence mineralisation processes in vitro or to have the potential to influence such processes. A number of studies have sought correlations between particular factors and either calculus or caries; almost none involved both.

Which trend to expect for each factor is not always obvious. Table 5 summarises the trends predicted on the basis of the mechanistic information reviewed in previous sections. For example, high levels of salivary calcium and phosphate and high salivary pH might be expected to be characteristics of high calculus-formers and/or caries-resistant people, because such conditions favour crystal growth. In contrast, high levels of salivary phosphoproteins (statherin, PRPs, CCPs) might be characteristic of low calculus-formers and/or caries-resistant people, because such molecules could well inhibit both crystal growth and dissolution. In principle, the phosphoproteins could inhibit the remineralisation of early carious lesions but this is less likely to happen in practice because the molecules are relatively large and may have difficulty in penetrating the surface zone.

Different authors often appear to have obtained conflicting findings concerning particular factors. Certain differences may be attributed to the use of a small number of subjects in a study who may not have been representative of the true population. Frequently, however, differences could have occurred because of natural changes in salivary composition resulting from diurnal variations, the effect of flow stimulation and, possibly, subject age. For example, it is noteworthy that, although salivary calcium and phosphate concentrations change relatively little with salivary flow rate, the protein content of saliva increases markedly with increasing flow rate [50]. A further complication is the

Table 5. Predicted effects of various salivary and plaque factors on calculus and caries

Factor	Calculus	Caries
Saliva		
Calcium	+	–
Inorganic phosphate	+	–
pH	+	–
pyrophosphate	–	?
statherin	–	–
proline-rich proteins	–	–
cysteine-containing proteins	–	–
histidine-rich proteins	–	–
mucins	?	–
IgA	?	–
Lipids	?	?
Acid phosphatase	+	+
Lysozyme	–	–
Peroxidase	–	–
Urea	+	–
Sialin	+	–
Plaque		
Base-producing bacteria	+	–
Acid-producing bacteria	–	+
Calcifying bacteria	+	?
Alkaline phosphatase	+	+
Protease	+	+

+ = high amount favours formation.
– = high amount inhibits formation.
? = effect uncertain.

timing of saliva or plaque sampling relative to that of the clinical diagnosis of caries and calculus. Caries especially is a disease that takes a long time to develop and the initiating oral conditions may have occurred many months before a lesion becomes clinically detectable.

1.5.1 Calcium and phosphate

There are numerous early studies of salivary calcium and inorganic phosphate in groups of caries-free and caries-active people, almost all of which

showed higher average concentrations of the ions in caries-free people [75]. However, statistically significant differences were rarely attained. More recent studies are, if anything, less consistent. Shannon and Feller [76] found the anticipated inverse relationship between parotid salivary calcium and phosphate levels, and caries rates. Furthermore, Shaw et al. [77] found a significantly higher average phosphate concentration in submaxillary–sublingual saliva from a group of caries-free children than in saliva from a group with high caries, although they found no difference for salivary calcium. In partial contrast, studies reported by Mandel [78] on salivary calcium and phosphate and by Turtola [79] on salivary calcium showed no significant differences between caries-free and caries-active groups.

A few authors have investigated the possible association between calcium and phosphorus levels in plaque and caries, and obtained reasonably consistent findings. Schamschula with various co-workers [80, 81] found the intuitively expected inverse association between plaque calcium and caries experience in two independent studies. In one of the studies they also found a significant inverse association for plaque phosphorus. Similarly, Shaw et al. [77] found significantly more calcium and phosphorus in plaque from caries-free children than in plaque from caries-active children.

Ashley and Wilson [82] reported inverse relationships between 3-year caries increments and levels of calcium and inorganic phosphorus in the plaque. Of importance, these authors were able to show that the time relationship between caries diagnosis and plaque sampling appeared to be crucial. They had taken both caries and plaque measurements yearly during a 3-year clinical trial. Plaque calcium and phosphorus data correlated significantly with the 1-year DFS increments obtained one year later, but did not correlate with the corresponding 1-year DFS increments obtained one year earlier.

The situation concerning a possible link between salivary calcium and inorganic phosphate, and calculus formation is similar to that described above for caries. In his 1969 review, Schroeder [1] cited a number of studies in which salivary calcium and phosphate had been compared for groups with and without dental calculus. The majority showed a tendency for saliva taken from calculus-formers to contain higher amounts of the above species, as anticipated, irrespective of whether analyses were of stimulated or of unstimulated saliva. However, differences rarely achieved statistical significance, as was the case with corresponding caries comparisons.

A notable exception was a study by Mandel and Thompson [83] who found no consistent differences between the respective calcium and phosphorus levels in both parotid and submaxillary salivas from groups of calculus-formers and non-formers. However, a later analysis of a number of salivary components in heavy and light calculus-formers by Mandel [84], showed that

the calcium content of submaxillary saliva was significantly higher in the heavy calculus group.

Mukherjee [85] collected whole saliva from 7 caries-prone subjects and 9 calculus-formers and observed a significantly higher rate of calcium phosphate precipitation in the samples from the calculus-formers. However, the validity of the experimental procedure can be questioned.

Poff et al. [86] reported a weak correlation between salivary calcium and calculus formation in their study of 15 individuals, but found no significant correlation between salivary supersaturation with respect to any of the calcium phosphates (calculated from inorganic composition analyses of both stimulated and unstimulated salivas) and the rate of calculus formation observed clinically. Larsen et al. [65] later attributed these findings to the intrinsic wide variation in salivary composition determined.

Konig [42] undertook an interesting dietary study with rats. A high sucrose, low phosphate diet (a) led to high caries with a low level of calculus, whereas a low sucrose, high phosphate diet (b) gave low caries and moderate levels of calculus. When diet (a) followed diet (b) some of the calculus deposits appeared to dissolve, presumably because of the increased production of plaque acid.

In summary, the data for calcium and inorganic phosphate levels in saliva and plaque tend to support an inverse association with caries and a direct association with calculus, as predicted on mechanistic grounds. However, statistically significant trends are rare, which suggests that these factors alone cannot account for the inverse relationship between caries and calculus.

1.5.2 Oral pH Factors

Early studies, where plaque pH was measured before and after in vivo rinses with sucrose or glucose (i.e. the generation of Stephan curves), provided convincing evidence that low plaque pH was related to caries activity [87, 88]. Average differences of about one pH unit were observed between caries-free subjects and subjects with high caries experience. In other studies, however, average plaque pH differences between low and high caries groups have been less marked. Rosen and Weisenstein [89] obtained a statistically significant difference in minimum pH of 0.2 units, whereas Manly et al. [90] obtained a similar, but non-significant difference.

The latter authors believed that their well-controlled study, where test solutions were applied to plaque samples in vitro, reflected the true difference between high and low caries groups better than the earlier in vivo studies. An alternative, and mechanistically more attractive, explanation for the greater discrimination achieved in vivo is that, in the mouth plaque acid is continuously subjected to the buffering, dilution and clearance effects of saliva. Saliva may be

as important a discriminating factor as plaque. This hypothesis is supported by the study of Abelson and Mandel [69], which showed that saliva in caries-resistant subjects neutralised plaque acid after an in vivo sucrose challenge significantly more rapidly than saliva of caries-susceptible subjects. Plaque pH minima in the two groups were comparable when the plaque was denied access to saliva.

Overall the number of studies, which clearly demonstrate differences in oral pH between caries-free and caries-prone subjects, seems surprisingly small. Furthermore, it is not possible to rank in importance the possible pH-modifying salivary components identified by Jenkins: water, bicarbonate, urea, sialin, and antibacterial factors [91]. However, certain authors have concluded that caries-free individuals appear to regulate the pH of their dental plaque more effectively than caries-susceptible individuals [92].

We are unaware of any investigations that link salivary pH effects and calculus directly. Epstein et al. [93] observed that patients undergoing hemodialysis had lower salivary flow rate, markedly increased salivary urea concentration and formed more heavy calculus than healthy individuals. The authors were unable to relate the higher calculus formation to those specific changes in saliva, since elevated levels of phosphate may also have contributed. However, the results of the animal dietary study of Konig [42], discussed in section 1.5.1, are consistent with low plaque pH favouring low calculus and high caries. Moreover, MacPherson and Dawes [60] demonstrated that the most calculus prone sites in the mouth are characterised by rapid sugar clearance and high salivary film velocity. They deduced that Stephan curves in plaque in such regions would tend to be less deep and of shorter duration, leading to 'a reduced tendency for acid dissolution of early foci of calcification'.

1.5.3 Salivary Protein

Until the 1970s dental researchers were unclear as to which of the salivary proteins were potentially able to influence mineralisation processes. Sometimes, in hindsight, the wrong proteins were studied or, more commonly, proteins tended to be grouped together as a class. Consequently, comparative studies tended to be uninformative. For example, Mandel and Thompson [83] found higher amounts of protein in saliva from heavy calculus-formers than from non-formers but could only speculate that a particular protein fraction might have been the source of the difference. In his 1974 review of the roles of saliva and plaque in caries, Mandel concluded that the relatively few reported studies of individual proteins had been essentially negative [78].

The value of comparative measurements of proteins in the saliva of subjects with different levels of caries or calculus is uncertain. Molecules that bind calcium, for example, may exert an influence on the processes in solution, but

others affect mineralisation processes by adsorption. In the latter cases, analyses of pellicle and of plaque for active fragments may be more meaningful.

This suggestion was made by Mandel and Bennick [94] after finding that the levels of proline-rich proteins were 'remarkably stable' in both parotid and submandibular saliva from subjects either with high or low calculus or with high or low caries. In an earlier study, Mandel [84] had found that, whilst small amounts of particular proteins differed between individuals, there was no consistent difference in amount between mixed saliva samples from heavy- and light-calculus formers. Anderson et al. [95] also reported that individual protein patterns were quite varied in parotid samples taken from either caries-free or caries-active adults, but again no significant difference was observed between the two groups.

Shomers et al. [96] cited the large variation in individual samples of submandibular–sublingual saliva as the reason for their finding of a similar average content of cysteine-rich phosphoproteins in groups of caries-resistant and caries-susceptible subjects.

The only authors to study the more relevant protein adsorption characteristics to date have been Ruan et al. [97]. However, they observed neither qualitative nor quantitative differences in adsorption from 1:1 mixed parotid – submandibular saliva samples, from 6 caries-free and 6 caries-active subjects, onto powdered enamel and cementum. The small number of samples compared may be the reason for a lack of discrimination. The authors did find that much more proline-rich protein and cysteine-containing protein were adsorbed to enamel than to cementum though they could not explain why.

A possible source of some of the variation in the above studies may well be degradation of salivary proteins by proteolytic enzymes in the mouth. Bennick et al. [98] found that pellicles formed in situ on pieces of enamel and more than 24 h old showed degradation of adsorbed proline-rich proteins, and that old pellicle from extracted teeth contained very little of these proteins. A more recent report from the same laboratory [99] described large numbers of small phosphoproteins in samples of whole saliva, which were mostly fragments of acidic proline-rich proteins and indicated rapid degradation. In apparent contrast, Lamkin et al. [100] found that the magnitude of proteolysis of whole saliva samples was much smaller than they had expected, albeit by an ex vivo adsorption technique.

A recent study by Ayad et al. [101] indicated a qualitative association between basic PRPs and caries: different peptides were found in the parotid saliva of 9 caries-free adults, who were life-long residents of Rochester NY prior to water-fluoridation, compared to those found in 9 subjects of similar age with high caries. The authors suggested that proteolytic processing of parotid salivary proteins differs between such groups.

No comparative studies of the large molecular weight mucins appear to have been done to date.

1.5.4 Lipids

Slomiany and co-workers [102–105] have conducted a number of studies of salivary lipids from different subject groups, all involving low numbers of subjects.

Parotid [103] and submandibular salivas [104] from three heavy calculus-formers contained 50% more lipid than salivas from three light calculus-formers. Lipids from the heavy calculus-formers included higher amounts of glyceroglucolipids, cholesterol esters and free fatty acids but lower levels of cholesterol and triglycerides. The proportions of neutral and acidic glycolipids were similar in both groups.

Similar analyses of saliva samples from 10 caries-free and 10 caries-susceptible adults [105] did not show the trend expected, if lipid content were a causal factor for the inverse association between caries and calculus. The lipid characteristics of the heavy calculus group appeared to be similar to those of the caries-susceptible group rather than the caries-free group. Saliva from the caries-susceptible group contained ca. 35% more lipid than saliva from the caries-free group and had more neutral lipids and phospholipids. The neutral lipids from the former group contained much more free fatty acids, triglycerides and cholesterol esters. The glyceroglucolipid content was similar for both groups.

A potentially more informative study concerned an analysis of lipids in pellicles from 8 caries-resistant and 8 caries-susceptible adults [102]. The total lipid content of pellicles from each group was similar, 22–24% of the dry weight, a much higher proportion compared with protein than found in saliva [105]. The glycolipids (all glyceroglucolipids) were the major lipid fraction, unlike in saliva where the neutral lipids predominated. The glycolipid content of both pellicle groups was similar but the caries-resistant group contained less neutral lipids and phospholipids, similar findings to those for saliva. The only apparent difference between the pellicle findings and the corresponding saliva findings was a higher proportion of cholesterol esters in 'caries-resistant' pellicle but a lower proportion in 'caries-resistant' saliva.

Of interest, lipid-containing pellicle was less permeable to lactic acid diffusion in vitro than lipid-depleted pellicle and pellicles from caries-resistant subjects were less permeable than pellicles from caries-susceptible subjects [102]. These observations suggest a possible protection mechanism against caries without highlighting the particular lipids responsible. Other studies have indicated that lipids adversely affect the association of calcium with salivary glycoprotein [105]. It has also been speculated that because lipids modify the hydrophobic nature of the pellicle, they may facilitate bacterial adhesion [102, 105].

1.5.5 Immunoglobulin A

A natural defence mechanism of the body against disease is the production of antibodies against bacteria. An antibody against oral bacteria, immunoglobulin A (IgA), is found in saliva. However, correlations between IgA levels to *S mutans*, the bacterium most closely associated with caries, and caries experience are inconsistent [106]. In his 1998 review of antimicrobial factors in saliva, Tenovuo concluded that naturally evoked salivary antibodies do not have enough power to influence caries development in adolescents and adults [107].

The reason for the above inconsistency may be in the nature of the immune response mechanism itself. Initially, bacteria become established in the mouth, potentially leading to caries. The presence of the bacteria stimulates IgA production, which, in turn, potentially leads to a reduction in bacterial population and hence reduced caries. The anticipated association between high IgA levels and low caries appears to be most marked in young children [108–110], when initial infection with bacteria occurs. Conversely, high IgA levels were associated with high caries in two studies of adults [111, 112], in whom bacterial populations were presumably in a more equilibrium state.

Ruan et al. [97] were unable to detect IgA amongst substances adsorbed onto enamel and cementum from saliva in vivo. This is perhaps not surprising since the mode of action of the antibodies is more likely to involve specific interaction with bacteria.

Any connection between salivary antibodies and calculus might be expected to be more tenuous, since calculus levels are not associated with any particular bacterium. The present authors are not aware of any study of such an association, though both IgA and IgG, an immunoglobulin originating in crevicular fluid, have been detected in regions of calcified deposits low in mineral [113].

1.5.6 Salivary Enzymes

Few studies have investigated links between salivary enzymes and caries or calculus. Neither Ruan et al. [97] nor Stuchell and Mandel [114] could find differences in lysozyme levels between caries-resistant and caries-susceptible subjects. On the other hand, Mandel [84] found significantly less lysozyme in submandibular saliva from heavy calculus-formers than from non-calculus formers, and a similar trend in acid phosphatase level was almost significant. Mandel speculated that lysozyme, by its interaction with cell walls [106], might modify initial calcification processes.

As noted in section 1.4 a phosphatase might be expected to influence calculus formation because it would break down naturally occurring pyrophosphate. However, salivary acid phosphatase is not the only enzyme able to do this; other phosphatases and pyrophosphatase originating in bacteria and soft tissue are also present in the mouth (see next section).

1.5.7 Specific Plaque Factors

Whilst a correlation between amounts of plaque and amounts of calculus has sometimes been demonstrated [115, 116], such clinical findings need not necessarily imply a causal link and certainly are not informative about the present discussion on calculus and caries. Indeed, clinical studies of calculus and plaque per se have tended to be concerned with the debate on the causes of gingivitis and periodontitis [117]. For information relevant here the results of more specific studies need to be examined.

Many studies have been done which focussed on particular plaque bacteria and caries [118], but these lie outside the scope of this report. However, the general conclusion that relatively high levels of acidogenic bacteria are associated with high caries experience is pertinent to the present discussion. Clearly plaques that are often at low pH are less likely to calcify than those that are not. Significantly, Sidaway [74] failed to identify *S. mutans* in a study of the microbial flora of mature calculus samples from 20 periodontitis patients despite finding up to 22 different bacterial species in a single sample. Sidaway remarked that this was in striking contrast to the comparable situation in dental plaque formed at the gum margin. The prevalence of lactobacilli, another acidogenic bacterium, was low but these bacteria usually only form a minor proportion of plaque flora. In contrast, *S. sanguis* type I/II was isolated from more calculus samples than any other streptococcus. *S. sanguis* species are ubiquitous in dental plaque but type I/II is not. *S. sanguis* type I/II produces ammonia from arginine, a process which would raise plaque pH and favour calcium phosphate precipitation. (By *S. sanguis* I/II, Sidaway may have been referring to what are now known as *S. gordonii* [119] – the species definitions and nomenclature of the Mitis group of oral streptococci have undergone frequent changes since his report [120]).

Driessens et al. [40] have discussed the above hypothesis, concerning the generation of a pH rise in plaque, in detail. They noted that high salivary urea levels have been found to correlate with calculus levels. High amounts of calcium and phosphate were measured in plaque after daily rinsing with a urea-containing solution [121]. The plaque of heavy calculus formers has been reported to contain higher levels of calcium and phosphate than plaque from light calculus-formers [122].

Of key importance, as noted earlier, plaque is likely to be a zone relatively free of crystal growth inhibitors. This is because of the presence of numerous enzymes able to break down both salivary pyrophosphate and salivary proteins. Watanabe et al. [123] observed a significant correlation between supra-gingival calculus scores and protein activity in saliva. They demonstrated that the protease originated in plaque bacteria [124, 125] and that the enzyme was able to nullify the inhibiting effect of salivary protein on calcium phosphate precipitation in vitro [125].

Edgar and Jenkins [67] observed that pyrophosphate concentration in plaque appeared to be inversely related to calculus formation. Bercy and Vreven [126] reported correlations between calculus and the activities of pyrophosphatase and phosphatase in saliva and plaque supernatant. Such enzymes have been found in plaque and in distinct regions of plaque overlaying calculus deposits [127], though not in calculus itself.

1.5.8 Summary

The above review of salivary and plaque factors, which could influence calculus and caries, indicates that consistent correlations between such factors and the extent of either condition are rare. Salivary calcium and phosphate concentrations appear to be the only variables that have been found to correlate with both conditions in the predicted direction. Oral pH would be expected to give the predicted correlations but the experimental evidence is limited. Many authors comment on the substantial overlap in observed distributions for particular factors between caries- or calculus-prone groups and corresponding non-prone groups. The generally broad distributions probably reflect genuine inter-subject differences but differences in saliva composition because of variations in sampling time, subject age, etc. may also have contributed. A further complication is that many salivary components are multifunctional. For many factors, of course, the appropriate studies have not been done. Tenovuo [107] in his review of antimicrobial factors in saliva suggested that 'overall antimicrobial capacity' may be more relevant than the presence of any single component. We conclude that the calculus–caries relationship will certainly only be explained in terms of a combination of factors.

1.6 Concluding Remarks

The results presented in section 1.2.1 indicate unequivocally that there is an inverse relationship between calculus and caries for similar-aged users of dentifrices with and without sodium monofluorophosphate (SMFP) as a source of fluoride. The magnitude of the dependence is of clinical significance. The mean percentage difference in 3-year caries increment between calculus-prone children and their calculus-free counterparts, 20.5%, is similar to the average caries reduction found for users of a 1000 ppm F SMFP dentifrice compared to users of a non-fluoride control dentifrice.

Since the results show a low level of calculus to be a good predictor of caries, baseline calculus status could be, and has subsequently been shown to be, a useful stratifying factor in subject selection for caries clinical trials.

The reason why many researchers have failed to find the above inverse relationship is most probably because their study subjects spanned a wide age range, in which the age dependence of both caries and calculus would be confounding factors.

Of the many salivary and plaque factors potentially influencing calculus and caries, only oral calcium and inorganic phosphate levels appear to make a significant independent contribution, in the studies reviewed in section 1.5. The lack of discrimination between caries- or calculus-susceptible groups and corresponding non-susceptible groups in many studies of potentially relevant factors, is likely to be because subject numbers were too small.

We believe that more scientifically rigorous investigations are warranted in order to understand better the key causal factors in the caries–calculus relationship. For example, moderately sized (but balanced) sub-groups of children should be chosen with different distinct calculus/caries characteristics. Their saliva and plaque should then be examined using the most appropriate proven methodologies for factors of interest. In order to account for the time dependencies of these factors, sampling would best be done on repeated occasions and, especially in the case of saliva, at consistent times of the day.

Acknowledgements

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The Structure, Function and Properties of the Acquired Pellicle

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2.1 Definition and Introduction

Formation of the acquired salivary pellicle is the result of biopolymer adsorption at the tooth–saliva interface. The term acquired pellicle was first suggested in a review of the nomenclature of the enamel surface integuments by Dawes et al. [1], to describe the cuticular material formed on the enamel surface after eruption. The pellicle consists of adsorbed proteins and other macromolecules from the oral environment (saliva, crevicular fluids) and is clearly distinguished from the microbial biofilm (plaque) (fig. 1).

This review covers the formation, composition, structure, function and properties of the acquired pellicle. Specifically, the formation of pellicle is considered in terms of thermodynamic and kinetic aspects. The composition of the pellicle is reviewed in terms of the proteins, carbohydrates and lipids that have been identified using a range of analytical techniques. The ultrastructure of the pellicle is described in some detail from studies involving enamel slabs carried in the mouth, in which the subsequent pellicle was analysed by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). The function of the pellicle is outlined in terms of its lubrication properties, its ability to act as a semi-permeable membrane and its overall protection of the underlying enamel surfaces. Since pellicle is formed at the interface between the enamel surface and the oral environment, the important process of bacterial attachment to the pellicle surface is described and the specific bacterial binding sites found in the pellicle are summarised. The influence of diet and nutrition on the pellicle layer is considered. The formation of extrinsic stain is discussed: in particular, the role that chlorhexidine
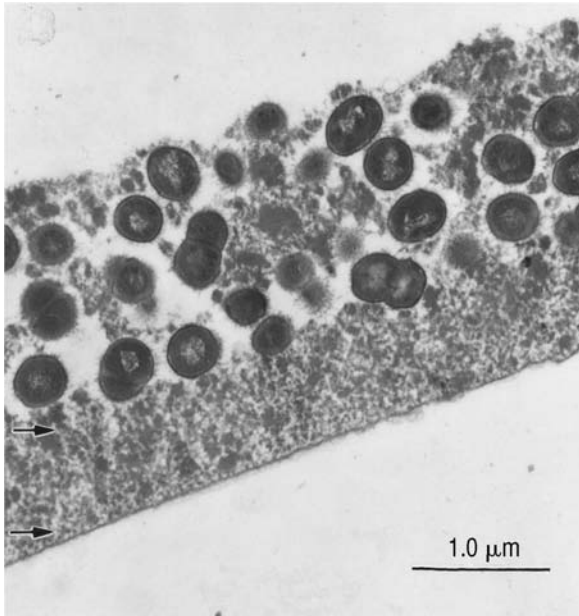
Fig. 1. TEM image of the acquired salivary pellicle layer (arrows) and adherent microbial biofilm formed in situ within 24 h on the enamel surface.

can have in promoting stain formation, together with possible mechanisms involved in non-chlorhexidine-induced, naturally formed stain. Finally, the influence that dental products can have on pellicle is examined.

2.2 Formation of the Pellicle

Pellicle formation is determined by adsorption of components from saliva, crevicular fluid and bacteria onto the enamel surface [2–13]. Formation of the acquired pellicle is a highly selective process, since only a fraction of the proteins available in saliva is found in the pellicle [14, 15].

The first stage of pellicle formation is characterised by an almost instantaneous adsorption of salivary proteins on the enamel surface. This initial adsorption process starts within seconds and probably takes a couple of minutes to be completed [13, 16–19]. The thickness of this initially adsorbed layer ranges between 10 and 20 nm [17] (fig. 2).

The first level of pellicle formation may be explained by the adsorption of discrete proteins onto the enamel surface. In contact with the watery electrolyte
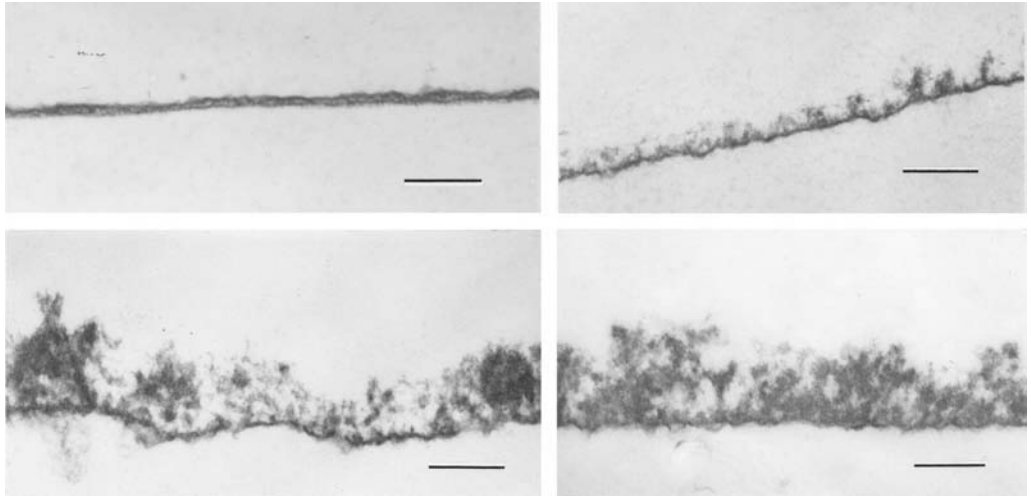
Fig. 2. TEM micrographs of the 1-min (top, left), 10-min (top, right), 60-min (bottom, left) and 2-hour pellicle layer (bottom, right) formed in situ on enamel specimens mounted at the buccal surface of the upper first molar. The adsorbed pellicle manifests itself as an electron dense, 10- to 20-nm thick surface layer, on top of which a second layer of globular appearance is formed. Formation of the outer, loosely structured pellicle layer increases with longer times of intraoral exposure. During processing for TEM investigation the enamel has been decalcified and is not visible in the micrographs. Bar = 200 nm.

solution of saliva, the calcium ions of the enamel crystallites have a stronger tendency than the phosphate ions to dissolve in saliva. As a result, the remaining phosphate ions will give the enamel surface an overall negative charge. This surface will be coated with a positively charged layer of calcium counter-ions. Thus, salivary proteins will be primarily adsorbed at the enamel surface due to electrostatic interactions between the ionic double layer (calcium and phosphate ions) on the one hand, and correspondingly charged groups of the proteins on the other [20–22] (fig. 3).

Known salivary proteins that exhibit high affinity to hydroxyapatite are called 'pellicle precursor proteins' [23]. Precursor proteins of the salivary pellicle derive from glandular and nonglandular secretions which are components of whole saliva. Phosphoproteins with a high affinity to hydroxyapatite, such as statherin, histatin and proline-rich proteins (PRPs), have been shown to be among the first proteins which adsorb onto the hydroxyapatite surface from glandular saliva [20]. These proteins are capable of exchange reactions with the phosphate ions of the enamel [11, 14, 20]. However, it has been demonstrated
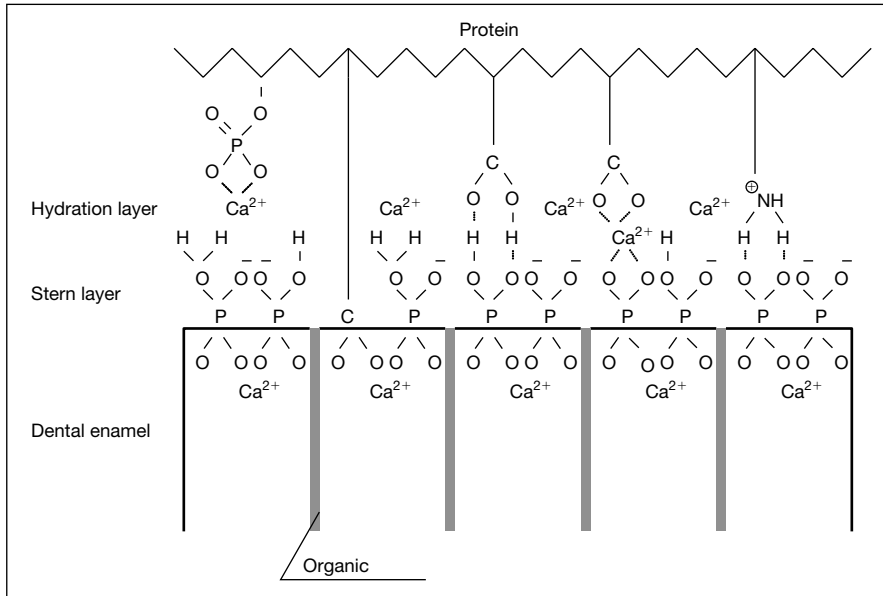
Protein

O O
\ /
P
/ \
O O
\ /
Ca^{2+}

Hydration layer   Ca^{2+} Ca^{2+} C   C   Ca^{2+} Ca^{2+} $\oplus$NH

Stern layer

Dental enamel

Ca^{2+} Ca^{2+} Ca^{2+} Ca^{2+} Ca^{2+}

Organic

Fig. 3. Schematic drawing of pellicle formation indicating adsorption of salivary proteins to the enamel surface via various electrostatic interactions.

recently that the initial pellicle layer formed in situ over periods of 30 s to 3 min reveals a more complex and heterogeneous composition than was considered previously. More than 10 different proteins have been detected and identified in the initially formed pellicle layer by electrophoresis and Western blotting techniques [13, 24], indicating the presence of mucin glycoproteins I and II, amylase, histatin, statherin, several isoforms of cystatin, lysozyme, lactoferrin, carbonic anhydrase I and II, as well as glucosyltransferase. Presence of phosphoproteins (PRPs, histatin and statherin) in the early pellicle is consistent with ionic interactions between proteins and the enamel surface. However, considering the fact that a broad range of different proteins has been identified in the early-formed pellicle layer, it is very unlikely that only ionic interactions determine the initial stage of pellicle formation. In addition to ionic interactions, other forces such as Van der Waals interactions will contribute to the adsorption of proteins onto the enamel surface, and hydrophobic interactions have also been suggested to play a role in the formation of the pellicle layer. These latter interactions are driven thermodynamically, as a result of a gain in entropy, which takes place when an adsorbing protein replaces structured water molecules at a surface [25, 26].

The rapid initial phase of salivary protein adsorption is followed by a second, comparatively slower phase of protein adsorption onto the protein-coated enamel surface. The second stage of pellicle formation is characterised by a continuous adsorption of biopolymers from saliva. This process involves protein-protein interactions between already adsorbed proteins, immobilised in the pellicle layer, and proteins as well as protein aggregates from saliva. Amino acid and Auger analyses of the pellicle layer formed on buccally carried enamel slabs [18] indicate that the adsorbed proteins reach an initial thickness in about 2–3 min, and stay at that level for about 30 min. The thickness of the pellicle then increases to about three times its initial thickness and reaches a plateau after 30–90 min [5, 18, 27]. Within 60 min, the thickness of the in situ-formed pellicle will further increase to between 100 and 1000 nm [17, 28], dependent on the supply of locally available salivary biopolymers and the prevailing intra-oral conditions [17, 28, 29] (fig. 2).

Concerning the rapid increase of the pellicle's thickness within 30–90 min, it is reasonable to postulate that the adsorption of protein aggregates rather than the adsorption of single proteins will contribute to the pellicle formation. It has been reported previously that most of the parotid proteins appear as globular structured aggregates with a diameter of 100–200 nm. These globules are suggested to have a hydrophobic interior and a negatively charged surface, and because of their similarity to milk micelles they have been called micelle-like structures [30, 31]. The amino acid profile of the micelle-like globules has been shown to be strikingly similar to that of the 2-hour in vivo pellicle layer [32], indicating that micelle-like globules present a major component of the newly formed pellicle. Recently, many heterotypic complexes between salivary mucins and other proteins from saliva have been identified, which are formed either before or after their adsorption to hydroxyapatite. These complexes involve noncovalent binding of amylase, PRPs, histatins, statherin, cystatins and lysozyme to MG1 [33], which suggests that the pellicle could be formed by adsorption of heterotypic complexes rather than single proteins. The enzyme transglutaminase, which is released in the oral cavity by buccal epithelial cells, seems to play a significant role in the formation of such bindings [33–35]. These heterotypic complexes are possibly a reservoir for pellicle precursor proteins and might protect salivary proteins from proteolytic degradation. In addition, these complexes probably function to concentrate proteins at interfaces. Micelle-like globules and heterotypic complexes can be described as supramolecular pellicle precursors [36].

The pellicle layer formed in situ over periods of 30–120 min reveals a knotted, globular surface texture with diameters of the adsorbed globule-like structures varying between 80 and 200 nm [37, 38]. Such observations also indicate that in vivo pellicle formation is mainly caused by adsorption of protein aggregates rather than by individual salivary biopolymers (fig. 4).
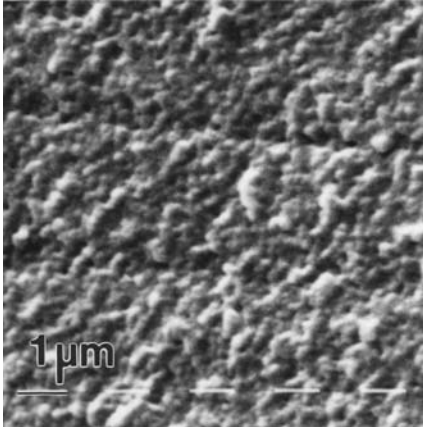
Fig. 4. SEM micrograph of the 2-hour pellicle layer formed in situ on the enamel surface.

Under in vivo conditions, enamel surfaces are continuously exposed to whole saliva as well as to the proteolytic activity of the oral fluid. Heterotypic complexing [33], enzymatic cross-linking [34, 35] or proteolytic processing can alter the properties of salivary proteins and lead to unique molecular species that will contribute to pellicle formation. Therefore, pellicle formation is a dynamic process [13] that is continuously influenced by adsorption–desorption processes, modification of adsorbed molecules by microbial or host enzymes and intermolecular complexing with other macromolecules. However, at the present time, data concerning the turnover rate of the acquired pellicle in vivo are lacking. In addition, there has been no clarification on the limiting factors during the development of the pellicle, and on the parameters that determine the equilibrium between adsorption and desorption in vivo.

2.3 Composition of the Pellicle

Studies on pellicle composition are hampered by the fact that only limited amounts (minute quantities) of pellicle material can be collected and recovered from human teeth in vivo for analytical investigations. It has been calculated that the pellicle layer formed per labial surface of a tooth over 2 h in vivo only contains approximately 1 µg of protein [39]. Therefore, much work has been performed using in vitro models to mimic the formation of the salivary pellicle. Glandular salivary secretions or whole saliva supernatants are used to form a pellicle-like protein layer adsorbed on hydroxyapatite or tooth enamel. Although considerable insight into the selectivity and affinity characteristics of salivary proteins during adsorption onto these surfaces has been obtained from

in vitro models, the in vitro-formed pellicle-like layer does not completely resemble the composition of the in vivo-formed pellicle [39–42]. Thus, information on the composition and the properties of the salivary pellicle layer in the oral environment can only be obtained by collecting pellicle formed in vivo [43].

In the 1970s, it was demonstrated that there were no systematic differences in the principal composition of the pellicle layer formed in different areas of the oral cavity [5]. Amino acid compositions of pellicles collected from the buccal sites of the upper molars, upper incisors and lower anterior teeth were found to be virtually identical [5]. In addition, amino acid analyses also indicate that the composition of the pellicle is consistent not only within, but also between individuals [12, 44]. A considerable intersubject consistency has been described concerning the composition of the in vivo-formed salivary pellicle layer [12, 39, 40, 45].

However, Carlen et al. [41] showed, by electrophoresis and immunoblotting, that the overall protein pattern of salivary pellicles from different parts of the dentition reveals characteristics that are typical for the saliva which may prevail in the area of the mouth where the pellicles were formed. These findings clearly indicate that the locally available salivary biopolymers are of importance for the formation of the pellicle layer and for its composition.

In recent years, improved methods of harvesting pellicle has been introduced that combine mechanical and chemical removal [39, 41]. The tooth surface is either swabbed with a polyvinylidene fluoride membrane filter soaked in $0.5\,M$ sodium bicarbonate [39] or rubbed with a foam sponge soaked with 2% sodium dodecyl sulphate solution [41]. Using these collecting methods, new data on the composition of the pellicle have been obtained [15, 39, 41, 46, 47].

2.3.1 Proteins

Several studies have been performed in order to determine the protein composition of the in vivo-formed salivary pellicle, using amino acid analysis and immunological, histochemical, chromatographic and electrophoretic methods [2–5, 7–13, 39–41, 46, 47, 48–52]. In general, these studies indicate that proteins and glycoproteins are the major salivary components of the in vivo pellicle. A large number of specific proteins involved in pellicle formation in vivo have been identified by the methods used in the above-mentioned studies and these are summarised in table 1.

Enzymes such as salivary α-amylase and lysozyme, as well as bacterial glucosyltransferases immobilised in the pellicle layer, have been shown to maintain their enzymatic activities [13, 50, 53, 54].

Plasma components, such as fibrinogen, fibronectin, albumin and IgG, have been detected in the pellicle formed in vivo [46]. These proteins were

Table 1. Proteins and other biopolymers identified as components of the salivary pellicle layer formed in situ/in vivo on enamel or hydroxyapatite

Biopolymer	Physiological role	Molecular weight (kDa)	Reference
Proline-rich proteins	pellicle precursor	5–30	9, 10, 41, 52
Statherin	inhibits precipitation of calcium	8	13, 15, 43, 52
Histatin	antimicrobial effect against candida species	3–5	13, 15, 43, 52
α-Amylase	digestion of starch	54–57	13, 37, 40, 41, 43, 47, 49, 50, 52, 53
Glucosyltransferase	bacterial enzyme (synthesises glucans)	140	13, 54
Carbonic anhydrase VI Carbonic anhydrases I, II	neutralisation of acids	42	13, 52, 55
Lysozyme	antibacterial effect (lysis of bacterial cell wall)	14	13, 43, 48–50, 52
Lactoferrin	iron-binding glycoprotein, antibacterial properties	80	13, 47, 48, 52
Mucin MG1	lubrication	>1000	13, 40, 43
Mucin MG2	lubrication	200–250	13, 41, 51
Cystatin SA – 1 Cystatin SN	antibacterial and antiviral properties	9	13, 39, 40
Albumin	carrier protein, regulation of colloid-osmotic pressure	69	39, 43, 46, 47
sIgA	immune response, inhibition of bacterial attachment	60/90	40, 41, 47–49, 52
IgM			48
IgG			41, 47–49, 52
Complement factor 3/3c	activation of the complement system	210 (80 and 130)	48
Fibrinogen	factor I of blood coagulation	340	41, 47, 49
Fibronectin	structural protein	2225	41, 47
Calgranulin B	calcium binding	13	15
Cytokeratin 13, 15	cell protein	40–67	15
Salivary agglutinin	agglutination of oral bacteria	300–400	41

Salivary amylase is found in the pellicle in two isoforms; the glycosylated isoform is present in the pellicle in larger quantities than the nonglycosylated form [40, 41].

found to a greater extent in pellicles formed at the gingival part of the tooth surface rather than at the incisal part [46]. Under healthy conditions of the gingival tissue, significantly more plasma proteins (albumin, fibrinogen, fibronectin) are detected in the pellicle layer formed on the gingival part of the tooth as compared to the incisal part of the tooth [46, 47]. Increased gingival crevicular fluid flow during gingivitis affects pellicle formation [47] and causes a significant increase of plasma proteins in the pellicle layer formed on the incisal part of the tooth surface. Again these findings clearly indicate that the locally available salivary biopolymers are of importance for the formation of the pellicle layer and its composition.

2.3.2 Recent Approaches for Protein Analysis of the Pellicle

Recently, MALDI-TOF (matrix-assisted laser desorption/ionisation-reflection time-of-flight) mass spectrometry was introduced as a new approach for the investigation of pellicle composition [39]. Using mass spectrometry for compositional analysis, it was found that more intact salivary protein species were present in an in vitro-formed pellicle compared to an in vivo-formed pellicle [39]. This finding suggests that the in vivo pellicle is an entity formed with components undergoing more extensive enzymatic (proteolytic) processing than in the in vitro pellicle. Therefore, in vitro-formed pellicle layers cannot completely mirror what occurs within the oral cavity [39]. This difference may be due to differences in the proteolytic capacity of the saliva supernatant used for in vitro pellicle formation and that of the oral environment. In addition, a particular saliva sample used for in vitro pellicle formation is a closed system, whereas the oral environment is an open system with continuous influx and clearance of oral fluids [39].

Analysis of in vivo-formed pellicle by a combination of electrophoretic separation and MALDI-TOF showed the presence of intact histatin 1, cystatin SN, statherin, lysozyme, albumin and amylase [15, 39]. In addition, intact cytokeratins 13 and 15 and calgranulin B were identified as components of the salivary pellicle layer for the first time using MALDI-TOF mass spectrometry [15]. Calgranulin B has been shown to be a component of saliva and gingival crevicular fluid [15]. The identification of cytokeratins in the salivary pellicle layer points to the oral epithelium as one of the sources of proteins adsorbed on the tooth surface.

Very recently, the preparation of monoclonal antibodies as a highly sophisticated approach to identify micro amounts of pellicle components has been described [43, 52]. Immunologic techniques are particularly suitable for identifying small amounts of antigenic components that provide sufficient immunogenicity to stimulate and induce antibody production by the immune system. Multiple encounters of the immune system with certain antigens amplify the

response with greater accuracy, and thus enable the detection of trace amounts of pellicle proteins. Immunisation of mice took place by intraperitoneal injection of human in vivo-formed pellicle material. Antibodies were obtained from the animals with reactivities against mucous glycoprotein 1 (MG1), PRPs, statherin, histatin 1, albumin, amylase, human sIgA, IgG, IgM, lactoferrin, lysozyme and carbonic anhydrase II, indicating that these components are present in the pellicle [43, 52]. The findings published by Li et al. [43, 52] indicate that all these biomolecules are integral components of the pellicle.

2.3.3 Carbohydrates

The pellicle has been found to contain several sugars, among which glucose seems to be the most abundant, accounting for more than half of the total amount of the carbohydrates [6, 56]. Mayhall and Butler [57] found that in vivo pellicle contains glucose and galactose in approximately the same concentrations. Also mannose, fucose, glucosamine and galactosamine have been detected in the pellicle layer [6, 56, 57]. The origin of glucose in the pellicle has been controversially discussed. It has been suggested that this sugar originates from salivary glycoproteins [57]. However, since these glycoproteins were found to contain only small amounts of glucose, this suggestion is rather unlikely. More likely, direct adsorption of glucose polymers (glucans) of bacterial origin will contribute to the glucose content detected in the pellicle layer in vivo. Most probably, glucosyltransferases adsorbed in the pellicle layer will synthesise glucans that will be the main source for the pellicle's glucose content. In addition, glycolipids, which are the major pellicle lipids, have also been proposed as a possible source of glucose, as the carbohydrate portion of the glycolipids consists of glucose only [58].

2.3.4 Lipids

The lipid content of the pellicle has not been thoroughly investigated, even though lipids seem to be a significant constituent of the pellicle formed in vivo [58]. It has been reported that lipids account for about 22–23% of the dry weight of pellicle. The major lipid classes identified in the pellicle are: (a) the neutral lipids, which are rich in free fatty acid, triglycerides, cholesterol and cholesteryl esters; (b) the phospholipids which have a high content of phosphatidylethanolamine, sphingomyelin and phosphatidylcholine and (c) the glycolipids, which consist of neutral and sulphated glyceroglucolipids. Interindividual differences in the lipid composition and content of the pellicle might reflect differences in the caries activity of the individual [59]. This observation has been explained by the ability of the lipid constituents to retard lactic acid diffusion through the pellicle layer, thus protecting the enamel surface from acids [58]. It has also been suggested that the ability of phospholipids to affect the physico-chemical

properties of the pellicle, by making it more resistant to acidic attack, is related to an increase in its tenacity due to interaction with mucins [59].

Regarding the apparent importance of lipids for the protective properties of the pellicle, it should, however, be kept in mind that the existing knowledge is solely based on two publications [58, 59]. Therefore, additional research is necessary to clarify the physiological role of the lipids involved in pellicle formation.

2.4 Ultrastructure of the Pellicle Layer

The prevailing knowledge on the ultrastructural pattern of pellicle formation and the micromorphological appearance of pellicle is mainly based on (conventional) transmission and scanning electron microscopic investigations [3, 17, 29, 60–67]. Only a very few results have been published using novel techniques, such as cryo electron microscopy [68, 69], CLSM [28], or atomic force microscopy [19, 38, 70] for analysis of the pellicle.

From early TEM studies, matured in vivo-formed pellicle has been described as a homogeneous and amorphous, bacteria-free surface coating of varying thickness [62–64, 67] (table 2). Less pronounced pellicle formation has been reported on lingual and labial sites of the teeth, as compared to the proximal areas [62, 63, 67]. In self-cleansing sites of the teeth, the pellicle thickness ranges between 30 nm and 80 nm [63], whereas in proximal areas the pellicle can be up to 2-μm thick [63].

More systematic SEM and TEM investigations of in vivo salivary pellicle formation at various time intervals indicate a more complex ultrastructural pattern of the adsorbed pellicle layer [66, 71–73]. The 'young' 2-hour pellicle has been identified as a 100- to 500-nm thick, uneven and incomplete organic coating of the enamel [6, 17, 66, 71]. After a period of several hours or days, 'maturation' of the pellicle results in the formation of a more compact layer with granular ultrastructural appearance [63, 71–73].

Most of these ultrastructural investigations were performed on enamel slabs carried in the buccal sulcus or mounted on the buccal sites of the teeth. However, within the oral cavity, saliva provides a series of distinctly different fluid environments [74], and secretions from different salivary glands vary considerably and have a specific protein composition [75]. Consequently, more recently published electron microscopic and CLSM studies have focussed on site-dependent differences in the formation (morphogenesis) of the in vivo pellicle [17, 28].

The salivary pellicle formed in situ within 1 min has been detected by TEM as a continuous, homogeneously structured, fine-granular, 10–20 nm thick, electron dense layer [17]. The subsequent adsorption of salivary biopolymers is governed by local influences of the oral cavity and is predominately dependent

Table 2. Thickness of in vivo formed 'old' salivary pellicle layer

Thickness (nm)	Reference
25–250	60, 61
100–200	62
30–80	63

'old' = Pellicle of unknown age but likely to be at least several months.

on the intraoral location of the tooth surface where the pellicle is formed [17, 28]. The 2-hour pellicle formed on buccal sites consists of a thin electron-dense layer and an outer loosely arranged layer, with both granular and globular components and variable thickness of 100–500 nm [17]. In contrast, the 2-hour pellicle formed on palatal sites is seen as a 20- to 80-nm thick, predominantly granular-structured layer [17]. Within 24 h, the thickness of the surface coatings adsorbed on palatal sites increases to 100–200 nm, and on buccal sites to 1,000–1,300 nm [17]. The 24-hour pellicle is characterised by an electron-dense layer on the enamel surface and above it a dense, homogeneous, granular-structured layer is observed. These time-dependent micromorphological changes of the pellicle can be viewed as a process of maturation, i.e. changes in composition and conformation of proteins in the adsorbed protein layer.

Amaechi et al. [28] analysed the thickness of the pellicle layer formed in situ within 1 h on enamel slabs in 8 intraoral sites using CLSM. After in situ pellicle formation, enamel slabs were stained by a combined anti-human immunoglobulin based on IgG, IgA and IgM (Ig GAM) conjugated to fluorescein isothiocyanate in order to facilitate visualisation of the pellicle in the CLSM. The CLSM results indicated that the pellicle thickness varied significantly between oral sites. Mean pellicle thickness ranged from 0.3 μm (palatal sites of the upper incisors) to 1.06 μm (lingual sites of lower molars). The thickest pellicle was formed at the lower posterior lingual tooth surfaces. These surfaces are constantly bathed in a pool of saliva from the submandibular and the sublingual glands. Correspondingly, the thinnest pellicle was formed at the upper anterior palatal tooth surfaces. These are poorly bathed in saliva and also are subject to the rubbing action of the tongue that is keratinised, and therefore might be more abrasive than the ventral surface [28].

These findings provide evidence for regional, site-dependent differences in the morphogenesis of the in vivo-formed salivary pellicle. It is suggested that factors acting locally in the oral cavity, shearing forces and the differing supply
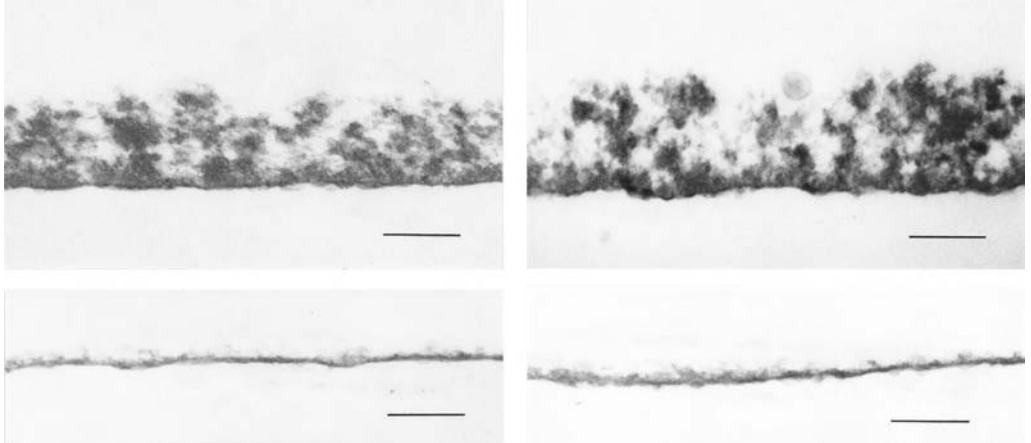
Fig. 5. TEM images of the 2-hour pellicle layer formed in situ on the surface (left) or within slots (right) of enamel specimens exposed to the oral environment on the buccal (top) or palatal site (bottom) of the upper first molar teeth. Comparison of the pellicle layers formed on the surface or within slots (i.e. shielded from shearing forces) does not reveal differences in thickness and ultrastructure of the adsorbed protein coatings. However, clear differences are evident between the pellicles formed within 2 h on buccally and palatally exposed enamel slabs. On the palatally carried enamel specimens – even within the slots – an outer globular pellicle layer cannot be detected. Bar = 200 nm.

of salivary biopolymers at different locations, determine the process of pellicle formation [17, 28, 29, 41]. Loosely structured globular pellicle layers, which are characteristic for the buccal sites of the dentition, probably are less resistant to the influence of shearing forces, and cannot be detected on the palatal site of the upper posterior teeth that are subjected to shearing forces due to tongue movement. In order to investigate the influence of shearing forces, enamel slabs with small slots measuring 1 mm in depth and 0.3 mm in width were exposed to the oral environment at the palatal and buccal site of the upper first molar teeth [76]. TEM analysis did not reveal any differences in the ultrastructural appearance and thickness of the 2-hour pellicle layer formed on the surface compared to the pellicle formed within the slots of the enamel slabs [76]. Neither on the surface nor in the slots of the palatally exposed specimens could an outer globular-structured pellicle layer be detected. These findings indicate that shearing forces resulting from tongue movement have no direct influence on the thickness and ultrastructural pattern of the adsorbed salivary pellicle layer (fig. 5).

In general, TEM investigations [17, 29, 71] indicate that the pellicle is composed of a tightly adsorbed electron dense basal layer and a loosely arranged outer layer. These ultrastructural findings are in good accordance with
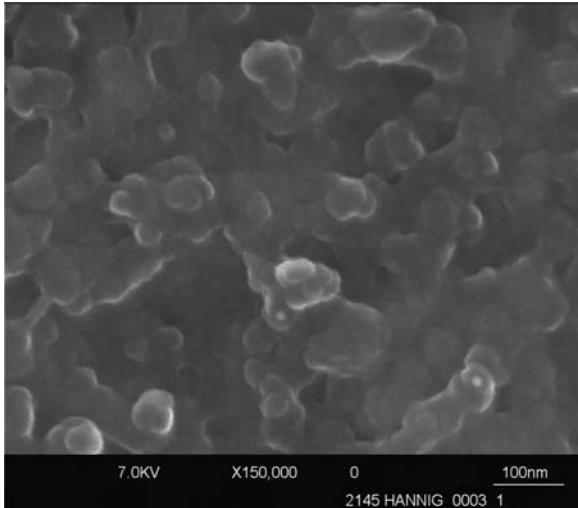
Fig. 6. High-resolution scanning electron micrograph of the pellicle layer formed within 30 min on the enamel surface.

previous observations of differences in the desorbability of adsorbed salivary proteins by buffer rinsing and elution using different types of surfactants [25, 26]. The results of these studies indicate that the salivary pellicle consists of loosely and firmly bound fractions of proteins that are attached to the enamel surface with different binding strengths [25, 26, 77].

Based on conventional electron microscopic analyses of fixed and dehydrated pellicle samples, an uneven, knotted morphological pattern of the pellicle's surface has been described [72, 73, 78]. Recently performed high-resolution SEM and atomic force microscopic investigations indicate that the surface of the in vivo-formed pellicle is characterised by a sponge-like network (meshwork) of spherical particles [19, 37, 38, 69, 79]. The diameters of these particles range between 10 and 20 nm within a few minutes of the initially formed pellicle [19], whereas in 30 min to 2 h pellicle layers much larger agglomerates of adsorbed proteins with diameters of 20–60 nm and even larger (100 nm) are detected [37] (fig. 6, 7).

The adsorbed pellicle layer probably consists of a random arrangement of diverse salivary biopolymers which are subject to certain changes dependent upon time. The variable ultrastructure of the (outer) pellicle layer reflects the complex processes of adsorption and desorption, which contribute to pellicle formation. Therefore, it is not possible to exactly describe the rate of pellicle formation or the final pellicle thickness. Nevertheless, from previous studies

0.5	1.0	1.5	μm
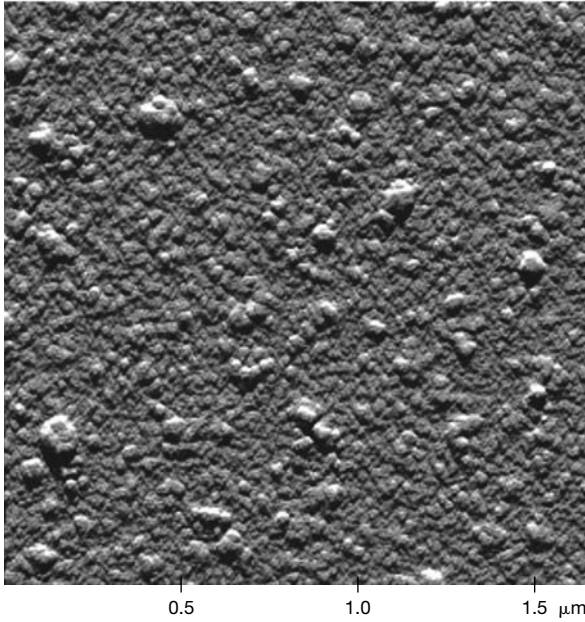
Fig. 7. Atomic force microscope image of the acquired pellicle layer formed on an enamel specimen mounted at the palatal site of the upper first molar and exposed to the oral environment for 10 min. The pellicle surface is characterised by a densely packed layer of globularly shaped particles of 15–30 nm diameter.

assessing the rate of pellicle formation, it has been concluded that the pellicle increases in thickness for about 1 h and that the rate of pellicle formation levels off between 1 and 2 h [18, 27].

2.5 Function of the Pellicle

The pellicle layer is of great physiological and pathophysiological importance for all interactions at the tooth–saliva interface. The salivary pellicle participates in all interfacial events taking place in the oral cavity, such as de- and remineralisation, lubrication of the tooth surfaces, and bacterial adherence.

2.5.1 Lubrication of the Tooth Surface
The tooth surface is lubricated by the pellicle, thus making mastication and speech more comfortable [80, 81]. The effect of lubrication has been

related to mucin glycoproteins I and II as well as statherin adsorbed in the pellicle layer [82–84]. High-molecular-weight mucins (MG1) have enhanced rheological properties, which lend these macromolecules to coating functions. The pellicle layer reduces friction between antagonistic teeth and between the teeth and mucosa. In a recently published study [85], the forces acting between adsorbed salivary films were measured by means of colloidal probe atomic force microscopy. It was found that the presence of adsorbed salivary pellicles between hard surfaces reduces the friction coefficient by a factor of 20. This reduction of friction is consistent with the long-range, purely repulsive nature of the normal forces acting between the salivary films. Thus, the lubricating mechanism is presumably based on a full separation of the sliding surfaces by the salivary films. This work suggests that the pellicle layer rather than the bulk salivary fluid is of more importance to lubrication than previously thought.

Moreover, it has been speculated that the pellicle layer protects the tooth surface against abrasive damage and excessive tooth wear [86, 87]. However, systematic investigations on the effect and influence of the salivary pellicle on tooth wear are lacking.

2.5.2 Semi-permeable Barrier

The pellicle acts as a barrier, which is important in maintaining the integrity of the enamel surface by preventing demineralisation and by facilitating remineralisation. In vitro-formed pellicle has been shown to possess a selective permeability function that regulates the de- and remineralisation processes at the enamel surface [88–90]. Due to its permselective nature, pellicle has the ability to modify acid diffusion and the transport of calcium and phosphate ions into and out of the enamel surface [58, 88–90], and thus is considered to play an important moderating role in the demineralisation of the enamel. However, the precise mechanism that regulates the diffusion of ions between the enamel surfaces and the oral environment in the presence of the pellicle layer is still not fully understood.

It has been suggested recently by Busscher et al. [91] that salivary pellicles are open structures allowing ion exchange at the enamel surface. This suggestion is in accordance with high-resolution SEM and AFM images of the pellicle layer [37, 69, 79], indicating a porous mesh-like surface pattern. Thus, the pellicle provides a medium through which fluoride, calcium and phosphate are delivered during recalcification. At the same time, it provides a certain protective function against demineralisation from microbial acid and against erosive challenges [88, 92]. The in situ-formed pellicle layer reduces and retards enamel demineralisation during acid exposure, but does not completely inhibit acid-related changes to the enamel surface [19, 93–95].
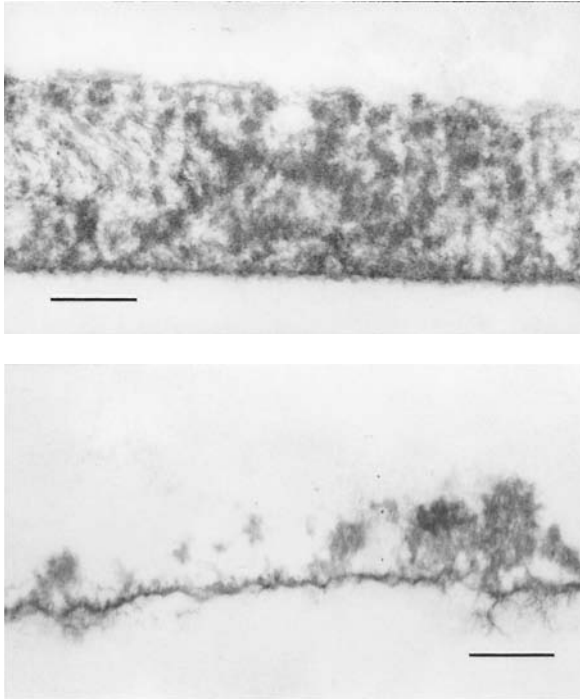
Fig. 8. TEM images of the 2-hour buccally formed pellicle layer (top) and the pellicle residues remaining at the enamel surface (bottom) after treatment with 0.1% citric acid over a period of 60 s. Bar = 200 nm.

Only a limited amount of data are available concerning the behaviour of pellicle and ultrastructural alterations of the pellicle during exposure to acidic agents [70, 93–95]. TEM investigations indicate that the in situ-formed pellicle layer gradually dissolves from its external to basal components during acid exposure [79, 93–95]. The less dense, loosely arranged outer pellicle layer reveals a comparatively high solubility in citric acid, whereas the densely arranged basal layer has a comparatively higher resistance against acid. Even after exposure to 0.1% citric acid for 300 s, the electron dense basal pellicle layer still appears as a continuous band without any interruptions at the enamel surface [79, 93, 94] (fig. 8). The fact that the pellicle is not completely removed from the enamel surface during short-term acid attacks suggests a partial acid resistance of the in vivo-formed pellicle layer. This, in turn, means that the pellicle might act to some extent as a diffusion barrier as well as a semi-permeable membrane [79, 95].

Pellicles formed at various intraoral sites are characterised by site-specific composition, thickness and ultrastructural appearance [17, 28, 41]. Protection against erosion has been related to the thickness of the pellicle layer [28], and evidence was provided for a direct relationship between the pellicle thickness of the in situ-formed 1-hour pellicle and its protective effects against erosion.

Not only the thickness of the pellicle and its ultrastructural pattern, but also the composition of the pellicle might contribute to its protective potential. Salivary carbonic anhydrase VI has been shown to accumulate in the enamel pellicle and still maintain its enzymatic activity [55]. In general, carbonic anhydrases catalyse the reversible reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Thus, it may be speculated that salivary carbonic anhydrase immobilised in the pellicle layer catalyses and accelerates neutralisation of acid, thereby protecting the enamel surface from demineralisation.

2.5.3 Importance of 'Maturation' for the Protective Properties of the Pellicle

It has been assumed from previous in vitro experiments [87, 89, 96] that the protective potential of the pellicle is enhanced with increasing formation time. These in vitro studies indicate that certain maturation processes with a duration of several days are necessary for the pellicle to get its full functional properties [88, 90, 96]. However, in recent years, conflicting results have been published regarding the influence of formation time on the protective properties of the pellicle layer. Although it is generally accepted from the results of in vitro studies, that the function of the pellicle as a diffusion barrier to ionic conductivity at the enamel surface will be improved with the process of pellicle maturation [88, 90, 96–98], recent in vivo studies have shown that even short-term in situ-formed pellicle layers are efficient in protecting the enamel surface [28, 95, 99]. It has been demonstrated that pellicle layers formed in situ over 2 , 6 , 12 , 24 h and 7 days do not differ significantly with regard to the protection of the enamel surface against erosion induced by citric acid [93–95]. Amaechi et al. [28] found that a 60-min in situ-formed pellicle layer gave some protection to the enamel surface against the erosive challenge of orange juice. A very recent study [99] indicates that even a 3-min in situ-formed pellicle layer provides a certain protective effect on the enamel surface against citric acid attack, that does not differ significantly from the protective effect of a 2-hour in situ-formed pellicle. These results indicate that maturation processes of a longer duration apparently do not enhance the protective potential of the in vivo-formed pellicle layer. Consequently, the processes of supposed pellicle completion and maturation might be of less relevance concerning the pellicle's protective properties

under in vivo conditions, as suggested from previously performed in vitro studies. This again confirms the importance of using in vivo- formed pellicle for studying processes occuring at the tooth interface where pellicle can play a major modifying role.

2.5.4 Mineral Homeostasis

Additionally, the pellicle modulates the process of mineral precipitation at the enamel surface. Saliva is supersaturated with respect to calcium and phosphate ions and provides the potential for remineralisation of the demineralised enamel surface. In the absence of the pellicle, calcium phosphate salts would continuously precipitate on the enamel surface [100]. Acidic PRPs reveal a high affinity for hydroxyapatite and are obligate components of the in vivo-formed pellicle layer [9, 10]. These pellicle proteins can act as inhibitors for surface-induced precipitation of calcium salts onto the enamel [101, 102], thus providing mineral homeostasis at the enamel–saliva interface.

2.5.5 Modulation of Bacterial Adherence

At the interface between the oral environment and the enamel surface, the salivary pellicle also exerts selectivity for bacterial adherence and is involved in the early stages of microbial biofilm formation (see below). Components of the pellicle serve as receptors for bacteria. Modification of the pellicle by host and bacterial enzymes may alter the functional characteristics of the pellicle surface, thereby influencing attachment of bacteria.

2.6 Pellicle and Bacterial Adherence

The oral cavity is colonised, in the main, by bacteria that are only found in the oral cavity [103]. The earliest bacterial colonisers of a freshly cleaned tooth surface appear within minutes [104] and tend to favour surface irregularities [70, 105]. Of the primary colonisers, streptococci predominate. *Streptococcus oralis, S. sanguis* and *S. mitis* account for 80% of primary colonisers [106]. In time, the adhesion of some of these initial colonisers becomes irreversible, so that their proliferation gives rise to sessile colonies. Coaggregation enables newly arrived species to colonise the surface of the pre-existing bacterial layer, an event that becomes more apparent after a few days of plaque growth [107]. As the bacterial layer increases in thickness, the initial, predominantly coccoid, Gram-positive colonisers are displaced by filamentous bacteria that eventually create an ecological environment favourable to the subsequent establishment of the predominantly Gram-negative, anaerobic microbiota [107].

In general, microorganisms are transported passively to the tooth surface by the saliva flow [108]. As a cell approaches pellicle-coated enamel, long-range physico-chemical forces provide a nonspecific attraction. The strength of this interaction is relatively weak, and this phase of attachment is reversible [108]. The strength of attachment can increase if the cell can get closer to the surface so that specific short-range stereochemical interactions can occur. Laboratory studies have also identified a number of specific molecular interactions that may be involved in adhesion to the tooth surface. It has been demonstrated that the adhesion of the initial colonisers to the tooth surface is mediated through a variety of structures that include pili, fibrils and fimbriae. The molecular components of these structures responsible for adhesion are referred to as adhesins [109] and include carbohydrate coatings, lipoteichoic acid, carbohydrate-binding proteins or lectins. These interact with a variety of receptors on the tooth surface, many of which are derived from salivary components [110, 111]. They can also interact with receptors on other bacteria, glycolipids or extracellular proteins such as collagen and fibrinogen [106, 112]. The receptors that have been determined in pellicle for oral bacteria are summarised in table 3. Thus, in general, pellicle components can promote bacterial adherence and initial plaque formation.

Progress in the characterisation of receptors found in pellicle that promote bacterial attachment to teeth has suggested that hidden molecular segments may be frequently exposed upon adsorption to the tooth surface due to conformational changes within the molecule. These hidden receptors are referred to as cryptitopes. Salivary components that resemble soluble receptors may interact with bacterial adhesins and block their attachment to teeth [134]. Cryptitopes, however, are not readily mimicked by related molecules in solution, and thus they evade host defences. It follows that adhesins which can recognise cryptitopes in surface-associated molecules would undoubtedly provide a strong selective advantage for an organism colonising a tooth surface. For example, studies have shown that acidic PRPs adsorbed to hydroxyapatite strongly promote adhesion of *Actinomyces viscosus* to the surface, whereas PRPs in solution do not strongly bind to this organism [133].

2.7 Influence of Diet/Nutrition on the Pellicle Layer

The adsorption of salivary components on enamel during pellicle formation has been shown to be a specific process that might be influenced by dietary components [13, 44, 65, 135, 136]. Some in vivo studies have suggested that differences in pellicle formation and composition are correlated with the dietary habits of the individual [44, 135]. The chemical composition of a

Table 3. Bacterial binding sites in pellicle

Bacterium	Binding site	Reference
Streptococcus mutans	Glucan	113
	Acidic proline-rich proteins	114
	High MW mucins	115
Streptococcus sanguis	Complex of secretory IgA light chain and α-amylase	116, 117
	α-amylase	118–120
	Fibrinogen	121
	Lysozyme	122
	Salivary mucin (MG2)	123–125
Streptococcus gordonii	Acidic proline-rich proteins	125
	α-amylase	120, 125–127
	Fibrinogen	121
	Salivary mucin (MG2)	124
	Glucan	129
Steptococcus pyogenes	Glucan	130
Streptococcus mitis	Fibrinogen	121
	α-amylase	131
	Glucan	130
Streptococcus oralis	Fibrinogen	121
	Salivary mucin (MG2)	124
	α-amylase	120
Streptococcus sobrinus	Glucan	132
Actinomyces naeslundii	Acidic proline-rich proteins	133
	Fibrinogen, fibronectin, IgG, IgA	46
Actinomyces viscosus	Salivary proline-rich proteins	33
	Proline-rich proteins, statherin	132, 133
Veillonella parvula	Glucans from glucosyltransferase	130
Fusobacterium nucleatum	Fibrinogen, fibronectin, IgG, IgA	46
Porphyromonas gingivalis	Fibrinogen, fibronectin, IgG, IgA	46

24-hour pellicle in comparision to a 2-hour pellicle changes after intake of a normal diet, but not in the case of fasting [44]. Acidic and neutral amino acids are most abundant and present in higher concentrations in the 24-hour pellicle formed while allowing diet intake as compared to pellicles formed within 2 h and 24 h whilst refraining from such intake. These differences indicate a dietary contribution to pellicle formation [44]. In addition, it has been shown by Vacca Smith and Bowen [135] that milk consumption as well as rinsing with sucrose, sorbitol or xylitol cause changes in the composition and formation of the salivary pellicle. In vitro studies indicate that milk proteins and casein derivatives can adsorb onto the tooth surface and are incorporated in the salivary pellicle in

exchange for albumin [68]. Thus, certain dietary components could be considered as an integral part of the pellicle [136].

2.8 Pellicle and Extrinsic Stain Formation

Pellicle has a tendency to develop stain, termed extrinsic stain, particularly in those areas of the dentition which are inaccessible to toothbrushing and the abrasive action of a dentifrice [137–139]. Extrinsic staining has been reported to be promoted by smoking, dietary intake of tannin-rich foods (e.g. tea, red wine) and the use of certain cationic agents such as chlorhexidine, tin and iron [140–144]. Proposed mechanisms of extrinsic stain formation have been reviewed over the years by a number of authors [140, 145–148] and the focus of recent attention has been the staining associated with chlorhexidine.

2.8.1 Chlorhexidine Stain

Chlorhexidine is a cationic bisguanide that is widely used in clinical dentistry as an antiplaque or anticariogenic additive for mouthwashes, toothpastes, varnishes etc., [149] and extrinsic staining of the tongue and teeth is a common finding following their use. This staining is of considerable interest to the dental profession and has prompted significant research effort in order to understand the mechanisms involved. Chlorhexidine is reported to promote the thickening of pellicle [150]. Pellicle collected from labial surfaces during chlorhexidine use is associated with an increase in calcification levels compared to pellicle collected when chlorhexidine is not used [67].

Recent debates concerning chlorhexidine staining have considered three possible mechanisms: nonenzymatic browning reactions (Maillard reactions), formation of pigmented metal sulphides of iron and tin and precipitation of dietary chromogens.

The nonenzymatic browning reaction mechanism, or Maillard reaction, occurs when amine-containing compounds and carbohydrates react to form coloured products [151]. Intermediate products in these reactions, such as furfurals, have been demonstrated in brown discoloured pellicle and in stained pellicle from subjects using chlorhexidine [152–154]. Despite these observations, the evidence for Maillard reactions in pellicle accounting for chlorhexidine staining is still inconclusive, an opinion expressed by Eriksen et al. [155] and reiterated by Watts and Addy [147]. However, in contrast, Nathoo and Gaffar [154] state that stain induced by chlorhexidine can be explained by an increased rate in nonenzymatic browning reactions occurring at the pellicle surface.

The second postulated mechanism involves the formation of pigmented sulphides of iron and tin within the pellicle. This is speculated to involve the

denaturing of pellicle proteins by chlorhexidine, and thus exposing sulphur containing functional groups. This leaves the possibility that metal ions, in particular iron and tin, could react with these to produce the sulphide salt of the respective metal [156, 157]. A study by Warner et al. [158] revealed increased levels of iron and sulphur in chlorhexidine-treated subjects compared with water controls and in subjects showing heavy staining compared to light staining. However, the authors concluded that the chromophore was not a metal sulphide but a complex between sulphur-containing organic materials and metal ions. The formation of pigmented metal sulphides via protein denaturation, and metal complexation within the pellicle, is rather a weak mechanism as argued by Addy and Moran [145] for the following reasons. First, dietary staining of chlorhexidine-treated tooth samples occurred regardless of the presence of pellicle. Second, dietary staining of saliva-coated tooth samples occurred only when specimens were exposed to chlorhexidine and not to known protein denaturants. Third, saliva-coated tooth samples exposed to protein denaturants or chlorhexidine did not stain when subsequently exposed to metal salts. This latter scenario was, in part, replicated in vivo where alternate rinsing with chlorhexidine and iron sulphate solution failed to produce staining of teeth in volunteers who abstained from food and beverages [159].

The third postulated mechanism involves the adsorption of dietary components into the pellicle. A number of in vitro experiments have demonstrated the ability of chlorhexidine to precipitate dietary chromogens from tea, coffee and a variety of beverages and food, to produce staining identical to that seen in vivo [160–162]. For example, salivary-protein-coated hydroxyapatite discs were either exposed to water, 0.2% chlorhexidine solution (2 min), black tea (5 min) or 0.2% chlorhexidine (2 min) followed by black tea (5 min) for two cycles of treatments. The amount of stain formed was measured by a reflectance chromameter and expressed as total colour change (ΔE) as shown in figure 9. This clearly demonstrates that water and chlorhexidine on their own do not form stain, and black tea forms some stain, but the combination of chlorhexidine followed by black tea forms significantly the most stain. The chlorhexidine/black tea effect has been confirmed by in vivo studies [163, 164], where subjects who drank tea or coffee along with twice-daily rinsing with chlorhexidine produced markedly more staining of the teeth compared with those who abstained from these beverages. In addition, subjects who rinsed their mouths regularly during the day with combinations of black tea and iron or black tea and chlorhexidine solutions developed stain, whereas those who rinsed only with tea, iron or chlorhexidine developed no stain [159]. It has also been observed that in vitro stain formation on Perspex specimens using saliva from different individuals with saliva/chlorhexidine/tea cycling occurred at differing rates when all other variables were standardised [165].
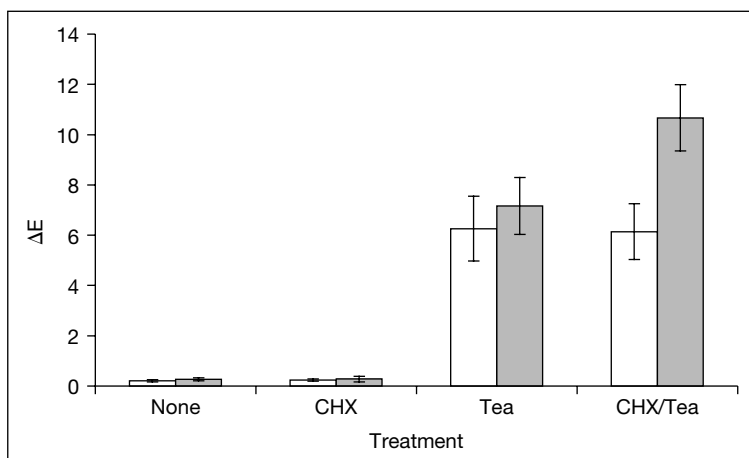
Fig. 9. Increase in stain on salivary-protein-coated HAP discs following one (white bars) and two (grey bars) treatment cycles.

The properties of the salivary pellicle accounting for this observation are currently unknown.

It is interesting to note that the colour formation caused by chlorhexidine/black tea interaction occurs after only short exposure times and this is speculated to be at a rate much faster than nonenzymatic browning reactions at body temperature. Thus, the overall evidence indicates that the most likely mechanism of staining by chlorhexidine involves the interaction of dietary chromogens with adsorbed chlorhexidine.

2.8.2 Natural Stain (Non-Chlorhexidine)

Evidence for metals (e.g. iron, copper, tin, silver, etc) causing stain formation appears to be significant only for individuals exposed to metals and their salts, typically in connection with their occupation or certain medicines [140, 147]. Stannous-fluoride-containing toothpastes have been reported to promote golden brown discolourations [63]. The mechanism for stain formation probably involves interaction of dietary chromogens and metal ions forming coloured complexes on the tooth surfaces, as indicated by in vitro and in vivo studies [159, 161].

In a recent in vivo study [143], the amount of extrinsic stain formed over a 6-week period of normal toothbrushing was found to be correlated with the amount of tea consumption and smoking. This confirmed the observation that smoking is well known to cause extrinsic stain [166], but also gave the

first non-anecdotal piece of evidence that tea components may be involved in extrinsic stain formation. The adsorption of components of black tea to pellicle has been studied using ellipsometric methods [167]. Pellicle exposed to black tea showed an increase in subsequent salivary protein adsorption compared to a non-tea-treated pellicle. Repeated black tea and saliva treatments showed a further increase in the amounts adsorbed with a concomitant increase in stain levels as measured by a reflectance chromameter. The stain was not removed by buffer or surfactant rinses, in contrast to a untreated pellicle. Similar results were found for red wine components [167]. In addition, the effects of black tea on the mechanical properties of in vitro-formed pellicle on human enamel have been investigated using a scratch-hardness technique [168]. Pellicles treated with black tea had a significantly higher scratch-hardness value as compared to a water control, indicating that black tea causes pellicle to become physically harder. Thus, black tea components have a profound effect on pellicle maturation, causing thickened layers of stained material to build up, which are not readily removed.

The role, if any, of nonenzymatic browning in non-chlorhexidine stain formation is unclear. Within the pellicle, there is certainly sufficient substrate of amine groups and carbohydrates for these types of reactions to potentially occur. The rate of this reaction is speculated to be relatively slow compared to the adsorption of dietary chromogens. Therefore, in order to investigate the rate of nonenzymatic browning reactions in pellicle, enamel blocks were placed on the partial dentures of a number of volunteers and worn in the mouth for 4 weeks in order to form in situ pellicles, as described by Joiner et al. [169]. The enamel blocks coated with 4-week-old pellicle were removed from the dentures and placed in individual vials containing either solutions of $0.1 M$ phosphate buffer (pH 8.0), 2% glucose in phosphate buffer or 2% glycine in phosphate buffer at 37°C. These conditions were chosen as increased pH, phosphate, amine sources (glycine) and reducing sugars (glucose) are known to increase the rate of the Maillard reaction. The colour of the samples were measured over 20 weeks with a reflectance chromameter in the Commission Internationale de l'Eclairage (CIE) L*a*b* mode and the change in b* (i.e. increase in yellow) was determined (fig. 10). Only a small increase in colour was observed for water and buffer controls. The addition of Maillard reaction substrates, such as glycine and glucose, did not cause significant colour changes until at least 12 weeks. Although not direct proof that Maillard chemistry is occurring, it does provide evidence that the rate of reaction causing general yellowing of pellicle through thermal ageing at 37°C is very slow. This rate is significantly slower than that observed for dietary chromogen adsorption to pellicle.
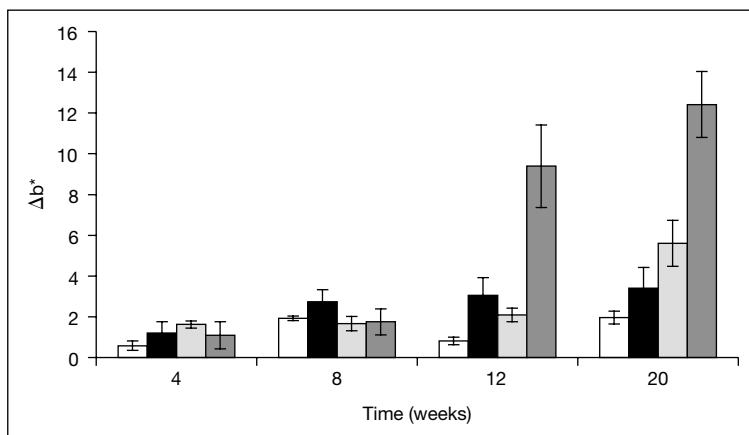
Fig. 10. Δb* values for in situ-formed pellicles stored in various solutions at 37°C for up to 20 weeks: water (white bar), buffer (black bar), glycine (light-grey bar), glucose (dark-grey bar).

2.9 Influence of Dentifrices on the Pellicle Layer

Only limited information is available from the literature concerning the behaviour of the pellicle during daily brushing of the teeth with a dentifrice. It has been reported from in vitro and in vivo studies that brushing with commercially available dentifrices significantly reduces the pellicle film quantity and its thickness [170–174]. Conversely, brushing with a nonabrasive dentifrice leads to a thick and often-stained pellicle [139, 175].

In a recently designed study, the effect of a silica dentifrice formulation on the ultrastructural appearance of the in situ-formed pellicle was tested [79]. Polished human enamel test pieces were placed into custom-made intraoral appliances and carried by adult volunteers over 6 h. Afterwards, the pellicle-covered enamel specimens were brushed ex vivo with a dentifrice–saliva slurry. The brushed enamel specimens were processed for TEM analysis in order to investigate changes in the pellicle's ultrastructure induced by the brushing procedure. Due to brushing with toothbrush and dentifrice, the 6-hour in situ-formed pellicle was reduced to a 1- to 30-nm thick residual basal layer without any adhering globular protein aggregates. In some of the analysed enamel specimens there were regions where no pellicle structures could be detected, indicating that the pellicle may have been removed in these areas due to brushing with the dentifrice. In contrast, brushing with artificial saliva frequently removes the outer globular pellicle layer and also causes partial reduction of the basal layer,

Table 4. Influence of fluoride treatment on salivary protein adsorption

Effect	Reference
Decreased adsorption of proteins	181
Desorption of adsorbed proteins by fluoride ions	182
Increased adsorption of proteins	183, 184
Reduction of protein-binding sites due to fluoride	184

however, without loss of the pellicle's continuity. The results of this study confirm previous findings indicating that the basal pellicle layer has a higher resistance against abrasion compared to the outer pellicle layer [173, 176].

After brushing, the pellicle rapidly reforms and provides protection to the enamel surface against acid attack even after a formation time of only 3 min [99]. Thus, loss of any of the pellicle during daily brushing of the teeth does not seem to have a major impact, and does not have any clinical relevance concerning pellicle-related protection of these enamel surfaces.

Professional tooth cleaning by the use of a rubber cup or rotary brush with pumice reduces old and matured pellicle to a great extent, but does not completely remove the pellicle from the enamel surface [177]. In the case of pumicing with a rubber cup, a continuous, few-nanometers-thick pellicle layer remains at the enamel surface, whereas after application of the rotary brush with pumice, a non-continuous, frequently interrupted residual pellicle layer is found [177].

In addition to the abrasive components of a dentifrice, other dentifrice ingredients could influence the pellicle and its formation. For example, pyrophosphate, which has been introduced in dentifrices to inhibit the formation of dental calculus, has been found to desorb pellicle proteins from enamel [178]. In addition, the commonly used dentifrice surfactant sodium lauryl sulphate has been shown to reduce the formation and coverage of the pellicle on enamel specimens [179].

Only a few studies have been published with regard to the influence of fluoride treatment on pellicle formation and composition. Contrary results have been reported concerning in vitro salivary protein adsorption on fluoride-treated enamel (table 4). Treatment of pellicle-covered as well as bare enamel surfaces with amine fluoride causes a decrease in surface free energy [184, 185]. However, fluoridation of the enamel surface with sodium fluoride does not significantly influence the amino acid composition of the pellicle layer adsorbed in vivo [186]. In contrast, stannous fluoride treatment of enamel

results in higher levels of acidic and neutral amino acids compared to the amino acid content of non-fluoridated enamel [187]. In addition, the pellicle layer is permeable to fluoride ions, and thus does not hinder fluoride uptake at the enamel surface [188–191].

2.10 Concluding Remarks

In conclusion, the formation of the acquired salivary pellicle is the result of biopolymer adsorption at the tooth–saliva interface and is a rapid process, starting within seconds in vivo. The pellicle layer is composed of components from saliva, gingival crevicular fluid, blood, bacteria, mucosa and eventually from diet. The pellicle is characterised by distinct structural diversity. The ultrastructural appearance and thickness of the pellicle are predominantly dependent on the intraoral location of the tooth surface on which the pellicle is formed. The pellicle should be considered as a dynamic system which undergoes continuous remodelling by the processes of adsorption and desorption, by chemical or enzymatic modification of adsorbed proteins, or through intermolecular complexing with additional proteins from saliva. The pellicle participates in all the interfacial events taking place in the oral cavity, such as bacterial adherence, de- and remineralisation, and in lubrication of the tooth surfaces. The pellicle layer plays an important role in maintaining tooth integrity by controlling mineral dissolution dynamics at the enamel surface and confers a certain resistance and stability against chemical dissolution and attack by acidic agents.

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Model Parameters and Their Influence on the Outcome of in vitro Demineralisation and Remineralisation Studies

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3.1 Introduction

In vitro models are probably the most widely used method of studying the de- and remineralisation of enamel. Their main strength is that experimental conditions can be very well defined and subsequently controlled throughout the duration of a study, e.g. pH, flow-rate, or solution composition. As such, they are particularly well suited to experiments whose objective is to study a single process in isolation, where a more complex situation with many variables may confound the data. An obvious disadvantage is that they cannot easily simulate the complex situation in vivo. However, the use of in vitro models for such studies is widely accepted and although further discussion is beyond the scope of this review, the topic is addressed by several excellent papers [1, 2].

One means of bringing in vitro models closer to reality is pH-cycling. This approach was first used widely in the 1980s [3–5] and involves subjecting hard tissue samples to repeated cycles of de- and remineralisation. Typically, sections of enamel or dentine are exposed repeatedly to experimental solutions which simulate application of an active agent/system, e.g. a dentifrice slurry, a cariogenic challenge, usually in the pH range 4.5–5.0, and a neutral buffer (fig. 1). This represents the sequence of events in vivo where a subject has brushed with a toothpaste, possibly containing an active agent such as fluoride, then consumed fermentable carbohydrates, leading to bacterial acid production and subsequent neutralisation of the acid by saliva. pH-cycling models are useful tools for the rapid evaluation of large numbers of putative new anticaries systems. Specific modes of action can be studied in isolation, for example, antidemineralisation potential using sound enamel [6, 7], or a more comprehensive approach can be adopted in a system which allows net de- or remineralisation
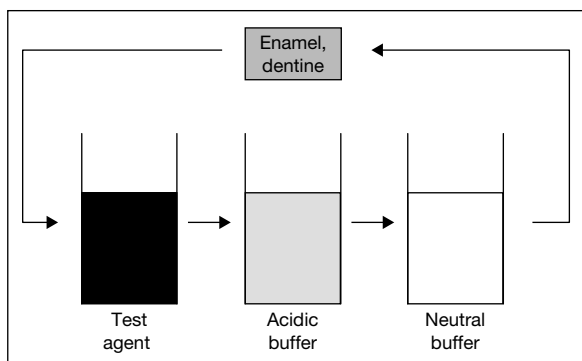
Fig. 1. Schematic representation of a typical 'pH-cycling' regime.

and hence utilises a partially demineralised enamel substrate [4, 8]. Further steps to improve the clinical relevance of these models have included the use of saliva or saliva substitutes [9, 10] and the addition of inorganic species to the experimental solutions in physiologically relevant amounts [11, 12].

The aim of this review is to look at some of the factors that can influence the outcome of in vitro studies into the de- and remineralisation of enamel and dentine. Intrinsic characteristics of artificial lesions, the effect of their environment, and the treatment regime to which they are subjected are all considered.

3.2 The Effect of Lesion Parameters at Baseline on Subsequent De- and Remineralisation during in vitro and in situ Studies

Several authors have reported that the extent of mineral loss in artificial lesions at baseline (ΔZ_{base}) can affect their subsequent behaviour when used to study de- and remineralisation in intraoral models [13–15]. Each found an increasing tendency toward net remineralisation with increasing ΔZ_{base}. The same effect has also been observed during numerous in vitro pH-cycling studies [16]. Figure 2 shows the relationship between ΔZ_{base} and the subsequent net mineralisation change of bovine enamel lesions observed during an in vitro pH-cycling study. As ΔZ_{base} increased, there was an increasing tendency towards net remineralisation.

This response to ΔZ_{base} is consistent despite a range of lesion preparation techniques and experimental protocols, both in vitro and in situ. For example, longitudinal analyses of both thin enamel lesion sections and bulk enamel
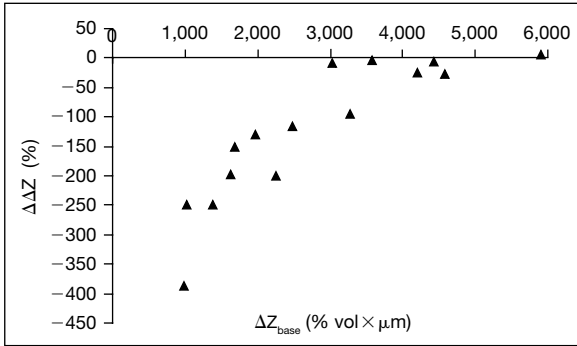
Fig. 2. Relationship between ΔZ_{base} and subsequent change in mineralisation, $\Delta\Delta Z$, observed in human enamel during a recent in vitro pH-cycling study. A negative value for $\Delta\Delta Z$ indicates net demineralisation, a positive value indicates net remineralisation.

blocks containing a whole lesion, from which a section is cut subsequent to treatment, have been used. There is evidence that thin sections behave quite differently to blocks [17], the former demineralising more rapidly than the latter. Further, in vitro, the response to ΔZ_{base} is consistent, regardless of whether human or bovine enamel is used. This suggests that the underlying cause is not some artefact of certain lesion preparation techniques, or of a particular experimental design, but some factor associated with pH-cycling or subsurface enamel lesion formation/progression.

Several explanations have been proposed. Mellberg [14] suggested that small lesions have the greatest absolute mineral loss during in situ studies because they are shallow, giving, bacterial acids easier access to material of relatively high solubility within the lesion, and that in large lesions, dissolution would be hindered by a longer diffusion pathway, i.e. small lesions are intrinsically more susceptible to further demineralisation. Schäfer et al. [15] reported that amongst smaller lesions, a few unusually soluble specimens skewed the data. Alternatively, Strang et al. [13] proposed that the greater porosity of larger lesions, leading to increased potential for remineralisation, is responsible. However, in all of these studies, the net change in mineralisation of lesions was assessed by analysis after repeated periods of de- and remineralisation. Thus, it was not possible to attribute the phenomenon to effects influencing demineralisation, remineralisation or both.

Lynch et al. [16] studied both in vitro de- and remineralisation of lesions independently, with a range of ΔZ_{base} values, in human and bovine enamel. They found a significant relationship between increasing ΔZ_{base} and decreasing tendency to demineralise, whether expressed as an absolute difference in ΔZ_{base} or

Table 1. Change in ΔZ after demineralisation of enamel lesions

Lesion type	ΔZ_{base} % volume $\times$ μm (SD)	n	% change (SD)*	$\Delta\Delta Z$* % volume $\times$ μm (SD)
Bovine	1060 (184)	5	−141 (41.3)	−1460 (345)
	2200 (408)	5	−56.8 (31.8)	−1230 (630)
	3670 (491)	4	−16.4 (11.2)	−570 (323)
Human	644 (119)	10	−245 (167)	−1489 (923)
	2110 (109)	5	−39.9 (23.6)	−825 (463)
	2800 (56.9)	7	−27.5 (21.5)	−774 (608)
	3320 (79.0)	9	−17.1 (9.70)	−568 (318)

* Negative values indicate further demineralisation, positive values indicate remineralisation.

as a percentage of ΔZ_{base} (table 1). The difference was most pronounced at the smaller end of the range of ΔZ_{base} values in both enamel types. Extent of remineralisation increased in direct proportion to ΔZ_{base} in bovine and human enamel. These findings support the suggestion that higher solubility of smaller lesions can affect overall mineralisation behaviour. In this case, the de/remineralisation equilibrium shifted towards net remineralisation via a reduction in mineral lost during the demineralisation phases of the pH-cycling regime.

Under remineralising conditions, the artificial lesions gained mineral in proportion to ΔZ_{base} (table 2). This would be expected; a larger lesion should have a greater internal surface area for precipitation of mineral than a smaller one. In the larger lesions, there was no apparent reduction in remineralisation through long diffusion pathways or high surface zone mineralisation, possibly because even the larger artificial lesions in this study were relatively shallow, and hence fairly open to ingress/egress of mineral ions. (Note that the concepts of 'shallow' and 'deep' lesions refer to lesion depth below the surface and not necessarily to extent of mineral loss; fig. 3.) Surface zone mineralisation was typically in the range 50–65% but never high enough to seriously impede remineralisation. However, high surface zone mineralisation can effectively inhibit remineralisation [18] and would clearly have an effect if sufficiently high.

An explanation for the relatively high solubility of smaller lesions may be differences in their chemical composition, when compared to larger lesions. In similar bovine enamel lesions, created in acid–gel systems over 5–15 days, considerable amounts of magnesium were lost [16]. This is consistent with the rapid loss of magnesium (and carbonate) during the early stages of natural caries [19–21]. Further, both species are associated with increased enamel solubility [22]. This difference may explain the trend towards decreasing lesion

Table 2. Change in ΔZ after remineralisation of enamel lesions

Lesion type	ΔZ_{base} % volume × μm (SD)	n	% change (SD)*	$\Delta\Delta Z$* % volume × μm (SD)
Bovine	1369 (418)	5	34.1 (23.0)	441 (232)
	2065 (142)	5	31.8 (12.0)	647 (218)
	3551 (553)	5	26.7 (18.8)	884 (557)
Human	689 (147)	10	24.4 (52.9)	224 (341)
	2458 (373)	7	23.7 (31.9)	598 (657)
	3029 (219)	6	15.8 (14.2)	475 (416)
	3317 (64.7)	7	25.7 (14.6)	866 (494)

* Negative values indicate further demineralisation, positive values indicate remineralisation.
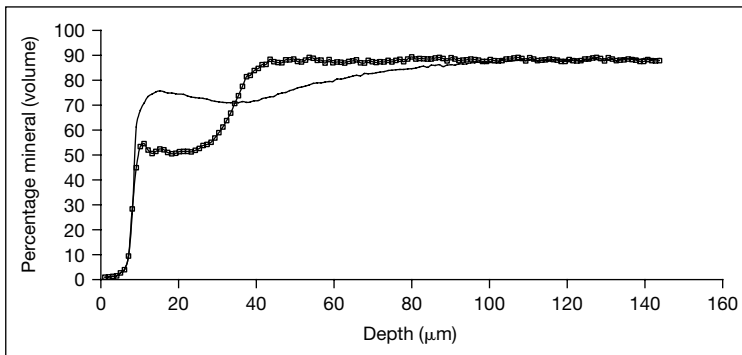
Fig. 3. Mean mineral density profiles of two artificial caries lesions. Mineralisation, as a percentage of sound enamel, assumed to be 87% mineral by volume, is expressed as a function of depth into the lesion. The two mineral distributions are clearly different but the amount of mineral loss is almost identical in each case. In the text, the terms 'shallow' and 'deep' refer to lesion depth, whereas the terms 'small lesion' and 'large lesion' refer to amount of mineral loss, regardless of depth. The heavy line represents a lesion with a high 'R' parameter and the lighter line, a lesion with a lower 'R' parameter (see 4.6).

solubility with increasing ΔZ_{base} and the more uniform response to de/remineralisation. Lesions of greater ΔZ_{base} in these studies were prepared by increasing the time spent in demineralising acid–gel systems, and preferential dissolution of more-soluble mineral associated with magnesium and carbonate should behind more acid-resistant (i.e., less soluble) material in the lesion body.

The relative merits of expressing changes in mineral content as a percentage of ΔZ_{base}, or as an absolute value, are worth discussing. Consider the case where no relationship exists between ΔZ_{base} and net mineral gain or loss
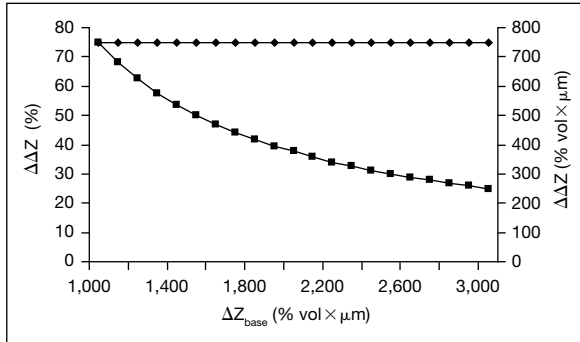
Fig. 4. Mineral loss data expressed as a percentage of ΔZ_{base} and in absolute terms (■ = percentage; ◆ = absolute).

i.e. mineralisation is independent of lesion parameters and proceeds simply as a function of time. Fig. 4 shows the effect of taking a range of ΔZ_{base} values and adding 750 units to each, representing net mineral loss during a hypothetical study, expressed as a percentage value and then as an absolute difference. When the data are expressed as a percentage of ΔZ_{base}, a strong relationship apparently exists between ΔZ_{base} and propensity to demineralise. When the same data are expressed in terms of absolute mineral change, no such relationship is seen. So, whilst data expressed in percentage form are useful when comparing effects in different substrates, for example, or when comparing substrates with a range of baseline values, caution should be exercised. It is probably better to use both means of expression if there is doubt as to which is more appropriate.

It is interesting to speculate on the effects discussed here in the context of in vivo lesion arrestment. Although ten Cate and Duijsters [3] suggested that this is the result of incorporation of large amounts of fluoride into the surface zones of lesions, it is possible that reduced solubility also contributes.

3.3 The Effect of Timing of Treatment Delivery during in vitro De- and Remineralisation Studies

Another approach to consider is the use of pH-cycling to assess the effect of timing, or mode of application, of an active agent relative to cariogenic challenges. This has direct clinical relevance. For example, a recurrent debate in the dental literature is on the relative efficacy of brushing the teeth before or after a meal. Brushing first may remove plaque, before it has a chance to produce acid, and also deliver beneficial active agents. Brushing immediately after eating

should remove plaque and food debris, but may also remove enamel softened by acid during the meal. Brushing some time after eating, when acid-softened enamel has had sufficient time to reharden through contact with saliva, has also been recommended. This may save enamel loss through abrasion but at this stage much of the residual carbohydrate from the meal will have been metabolised to harmful acids and obviously any active agent may be delivered too late to confer a benefit.

ten Cate et al. [23] varied the timing of fluoride applications to enamel in an in vitro pH-cycling study. Fluoride, at concentrations similar to those found in vivo after brushing with a fluoride dentifrice, was applied to the enamel either before or after an acidic challenge, with subsequent exposure to a neutral buffer. They reported that timing had no effect on reduction in enamel demineralisation. In a study with a similar objective, Lynch and coworkers used a more complex pH-cycling model [24] to study the effect of timing of application of calcium glycerophosphate [25]. The model was essentially a set of flow-cells, inoculated from a chemostat, which contained a bacterial consortium representative of the range of types found in the mouth and based on that used by Bradshaw et al. [26]. Sound enamel and dentine sections were mounted in each flow-cell and sucrose was pulsed in twice daily to provide a cariogenic challenge. A solution of calcium glycerophosphate was pulsed in either 1 h before the sucrose pulse, at the same time as the sucrose or 1 h after it. A control group was included, where no glycerophosphate was pulsed. The inserts were analysed radiographically subsequent to pH-cycling to assess the extent of mineral loss. In contrast to the findings of ten Cate et al. [23] relating to the effect of timing of a fluoride treatment, we found that timing had a marked effect on the efficacy of the calcium glycerophosphate. Pulsing in advance was significantly more effective at reducing demineralisation when compared to pulsing at the same time or after the sucrose pulse. For the dentine, application in advance was the only mode in which calcium glycerophosphate was able to significantly reduce demineralisation.

The most likely explanation for the pronounced difference in the effect of timing in these studies is a difference in the mechanisms by which fluoride and calcium glycerophosphate exert an antidemineralisation effect. In the former study [23], it was suggested that the strong fluoride–enamel interaction ensured that even when applied after an acidic challenge, sufficient fluoride was bound to the enamel to confer protection during subsequent acidic challenges. Conversely, the most likely effect by which CaGP reduces demineralisation, when delivered intermittently and at relatively low concentration, is elevation of biofilm calcium and phosphate concentrations [27], and this effect is at a maximum shortly after application in vitro [25] and in vivo [28].

The means by which fluoride reduces caries has been the subject of much discussion. Does a relatively short exposure to a high concentration of fluoride

exert the predominant effect? Or does the elevation of background salivary concentrations, typically from approximately 9 ppb when fluoridated oral care products are not used [29] to values in the range 14–25 ppb [30–31] many hours after the use of a fluoridated dentifrice, play the major role? Recent reviews of the subject have concluded that the latter effect is the more important, based largely on data generated using pH-cycling models designed to study the effect [32–34]. Page [35] used a model optimised for net demineralisation, to study the effect of intermittent delivery of fluoride at high concentrations compared to fluoride present continuously in all experimental solutions at very low levels. He concluded that fluoride continuously present at sub-ppm concentrations was as effective at reducing demineralisation as intermittent exposure to a dentifrice slurry containing fluoride at a concentration of several *hundred* ppm, and that even a concentration of 14 ppb reduced enamel dissolution by nearly 20% when present continuously.

So, it can be seen that when designing studies which utilise pH-cycling regimes, the timing of application of the active agent of interest must be considered carefully in the context of the objectives of the study. In the case of calcium glycerophosphate and dentine, the effect of timing was such that no significant reduction in demineralisation would have been found if timing had not been included as a variable, as the optimum potency of calcium glycerophosphate is relatively transient. In the case of fluoride, the same agent can exert the same effect at concentrations which differ by orders of magnitude by simply modifying the timing of delivery. Further study of these effects in vitro should lead to greater understanding of the mechanism of the anticaries agents discussed here, and elsewhere, hence facilitating the design of in vivo treatment regimes which optimise their benefits.

3.4 De- and Remineralisation under Simulated Plaque-Fluid Driving Forces

Many remineralisation models mimic the situation found on smooth enamel surfaces in the mouth, i.e. appropriate salivary calcium, phosphate and fluoride concentrations and a clean enamel surface with no plaque analogue. Remineralisation of enamel lesions typically occurs during exposure to experimental solutions at neutral pH, whereas demineralisation occurs during exposure to acidic buffers. However, when good oral hygiene is maintained and with the use of fluoride toothpastes, caries is prevalent not on the smooth surfaces of the teeth but on those sites which are difficult to clean and where plaque tends to accumulate i.e. occlusal fissures and approximal surfaces [36]. Here, caries is initiated and progresses not on a clean, smooth surface exposed to saliva, but

beneath a layer of plaque. Therefore, it is relevant to study de- and remineralisation of enamel lesions using experimental solutions representative of the plaque-fluid i.e. the extra-cellular phase of dental plaque.

There are several important differences to consider between the two types of site, when attempting to design physiologically relevant plaque-fluid models. Whilst free calcium concentration in plaque fluid is about the same, or less than, that in saliva, total calcium concentrations may be much higher [37–39]. Free and total fluoride concentrations are always likely to be tens or hundreds of times higher in plaque when all else is equal [40]. Rose et al. [41] have shown that model plaque biofilms can act as a reservoir for calcium and fluoride, releasing both species as pH falls during a cariogenic challenge. Frequency of acidic challenge is also likely to be a more important factor in realistic plaque-fluid models. Whilst the extent of demineralisation is dependent upon the duration and extent of undersaturation with respect to enamel [42], Pearce et al. [43] have shown that the ability of plaque to act as a mineral reservoir can be reduced with increasing frequency of cariogenic challenge through depletion of bound species. A further consideration is diffusion, which should be restricted in some way if the conditions found within occlusal fissures and the interproximal area are to be modelled.

Although the concept of plaque fluid is not new, it is only relatively recently that enamel de- and remineralisation under plaque-fluid conditions have been studied and reported in some detail. Exterkate et al. [44] showed that both the rate and site of remineralisation are different when comparing 'normal' remineralising solutions with one representative of plaque-fluid. Margolis and coworkers [45, 46] studied enamel demineralisation under plaque fluid conditions. They found that in solutions which were almost saturated with respect to enamel, even small differences in pH and calcium/phosphate concentrations could have a marked effect on dissolution rates. Blake-Haskins et al. [47] used an agarose gel as a plaque analogue to study the effect of varied plaque calcium and fluoride treatments on subsequent demineralisation. They found that a calcium rinse followed by a fluoride rinse was considerably more effective at reducing lesion size than fluoride alone. However, concentrations of both species were high when compared to reported in vivo values.

In a study to simulate conditions in plaque-fluid during an acidic challenge, Lynch et al. [16] placed pre-formed subsurface enamel lesions in a series of acidified methyl cellulose gel systems. The initial calcium, phosphate and fluoride concentrations of the gels were adjusted by the addition of calcium chloride, potassium phosphate and sodium fluoride. Two combinations of calcium and phosphate concentrations (high and low Ca,Pi), three pH values (5.2, 5.0 and 4.8) and four fluoride concentrations were used in a 2*3*4 factorial experimental design. Lesion mineral content was assessed radiographically before and
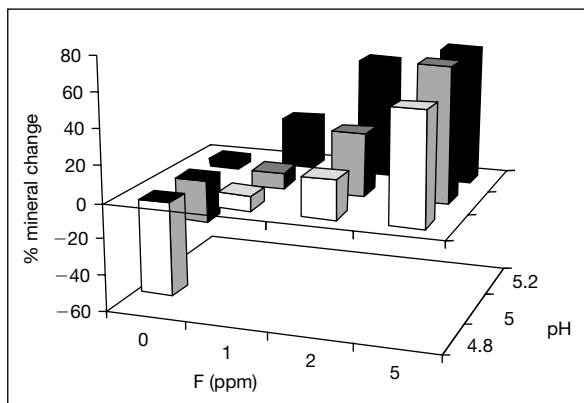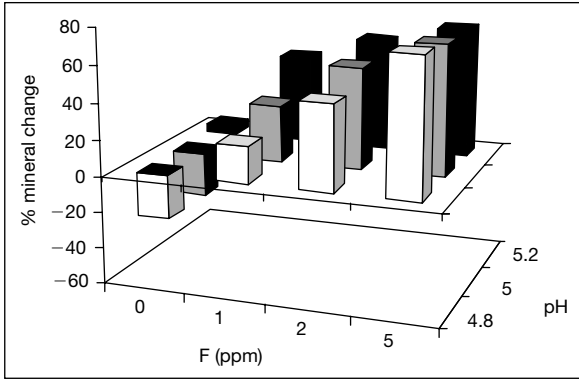
Fig. 5. Results of radiography, low CaPi group. A negative value for '% change' indicates net demineralisation, a positive value indicates net remineralisation.

after immersion in the gels. In this study, conditions in the acid-gel systems were intended to represent plaque-fluid conditions. pH values at and below 5.2 were chosen on the basis of the suggestion of Larsen and Pearce [48] that this is a better value for the critical pH of hydroxyapatite (i.e. the highest pH at which demineralisation can occur), rather than the more commonly used value of 5.5, which is based, arguably somewhat erroneously, on the classic studies of Ericsson [49]. The calcium and phosphate concentrations of the gels were based on the degree of saturation with respect to enamel $(DS_{En}) = 0.28$ and 0.36 demineralising solutions as defined by Zhang et al. [46], values both representative of plaque fluid concentrations and found to demineralise sound enamel at pH 5. The relative proportions of free and total calcium in the gels were similar to those of plaque fluid, with free calcium being a small fraction of the total calcium present. Calcium activity, determined by ion-specific electrode, was similar to that reported by Gao et al. [50] in the fluid of plaque harvested from caries-positive individuals, following a sucrose challenge. A wide range of plaque fluoride values have been reported [51]. The fluoride concentrations used here were similar to wet weight values found in resting plaque 12 h after the use of a fluoride toothpaste [32].

Figures 5 and 6 show the results of radiography for the low Ca,Pi group and the high Ca,Pi group, respectively. Whilst further demineralisation occurred in nearly all cases when no fluoride was added, in both Ca,Pi groups, significant remineralisation occurred in the groups with added fluoride. Secondary demineralisation was observed in nearly all lesions. In the case of the samples in the gels without added fluoride, this was the result of dissolution of enamel from the deepest part of the initial lesion and led to further net

Fig. 6. Results of radiography, high CaPi group. A negative value for '% change' indicates net demineralisation, a positive value indicates net remineralisation.
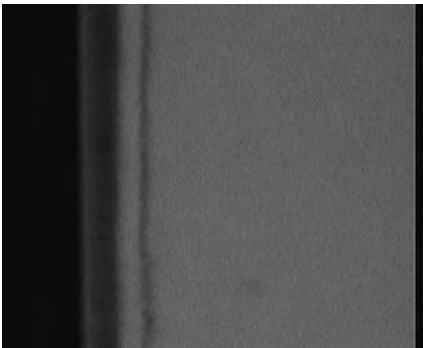
Fig. 7. Radiograph of a lesion remineralised at pH 4.8, 1 ppm F, low CaPi, showing pronounced lamination within the lesion body.

demineralisation. In the case of the lesions from the gels with added fluoride, the secondary demineralisation occurred beneath extensive remineralisation, and sometimes hypermineralisation, of the lesion body (fig. 7).

In this study, extensive remineralisation was observed at pH values usually associated with demineralisation, for example, in studies that simulated smooth surface sites in contact with saliva, using experimental solutions composed to reflect salivary calcium, phosphate and fluoride concentrations [8, 35]. This contrast highlights the need for further study of de- and remineralisation under plaque-fluid conditions.

3.5 Intra- and Intersubstrate Interactions during in vitro De- and Remineralisation Studies

Dissolving hard-tissue substrate will modify the composition of experimental solutions, with which it is in contact. This behaviour reflects the in vivo situation, where dissolved mineral from the teeth can reduce undersaturation during an acidic challenge.

In studies of enamel demineralisation in acid-gel systems initially infinitely undersaturated with respect to enamel [Lynch, unpubl. data], demineralisation began fairly rapidly after 3–4 days and then the rate reduced quite markedly after 14 days. Conversely, when enamel was demineralised in solutions which were just undersaturated and replaced every 3–4 days, demineralisation progressed in direct proportion to time over approximately 8 weeks. In the former case, mineral from the dissolving enamel would only have diffused away slowly, raising DS_{En} with progressive demineralisation until it led to a quantifiable reduction in the rate of dissolution. In the latter case, dissolving mineral would only have increased DS_{En} of the already partially saturated solution by a very small increment. Further, the solutions were replaced before any increase in saturation could have a substantial effect. In an aqueous, stirred demineralising system, where dissolved mineral from the developing lesion was removed rapidly into the bulk of the solution, Ruben et al. [52] found that lesion progression was more rapid than in an acid-gel system where diffusion of dissolved mineral into the bulk of the system is restricted.

Dissolution products from a developing enamel lesion in an acidified gel can also cause differences in the rate of demineralisation within that lesion, depending upon the relative ease with which they can diffuse away from the lesion surface [52]. In acid-gels, DS_{En} is higher above the edges of the lesion because dissolving mineral can diffuse away from the edges more easily. This enhanced dissolution at the edges results in 'flaring' in the lesion profile when viewed in section (fig. 8).

There has been some interest in recent years in the phenomenon described as 'hidden caries', and studies have been conducted to model processes occurring at the enamel–dentine junction (EDJ). ten Cate et al. [11] studied the effect of slightly elevated levels of fluoride, such as the typical salivary background concentration resulting from water fluoridation or use of fluoridated toothpastes, on the de- and remineralisation of enamel and dentine lesions. They concluded that slightly elevated fluoride levels may be considerably less effective in inhibiting lesion progression in dentine than in enamel, i.e. that differences in intrinsic solubility are important.

However, it has been reported that dentine can have a profound influence on enamel demineralisation when the two substrates are in close proximity, and

Fig. 8. Radiograph of a lesion showing characteristic 'flaring' at edges.

hence able to interact. During an in situ study [53], enamel and dentine sections were placed next to one another in an environment that restricted diffusion, and thus had the potential to localise dissolution products from the two substrates. Dentine dissolved to a greater extent than did the enamel, retarding demineralisation and facilitating remineralisation of the enamel during periods at higher pH, while the dentine underwent net demineralisation. The use of restricted volume conditions was a good analogue for the diffusion-restrictive conditions which would be found within a lesion at the EDJ.

Lynch et al. [54] reported findings from studies which examined the effect of placing sound and demineralised enamel sections next to dentine sections in acid-gel systems, which also restrict ionic diffusion. They found that enamel demineralisation was inhibited almost completely at pH 4.6 when enamel sections abutted directly onto dentine sections. Erosive, then subsurface, lesions formed within the enamel sections with increasing distance from the dentine, giving a characteristic 'wedge-shape' (fig. 9).

Further studies with similar, initially completely undersaturated gels (with respect to enamel) suggested that remineralisation might be possible and this was confirmed when pre-formed enamel lesions were placed next to dentine sections at pH 5, with gel calcium and phosphate concentrations adjusted to simulate plaque-fluid conditions. Significant remineralisation occurred in all enamel lesions. Control lesions in a similar gel but without added dentine demineralised further. One proposed explanation for this was an elevation of DS_{En} at the enamel/acid-gel interface resulting from the more-rapidly demineralising dentine sections in a situation analogous to those in acid-gel systems discussed earlier i.e. remineralisation at relatively low pH.

An alternative, or complementary mechanism could have been that low levels of fluoride from the more-rapidly dissolving dentine caused the effect, inhibiting enamel dissolution more effectively than that of dentine itself, as reported by ten Cate et al. [11]. Although fluoride concentrations measured in
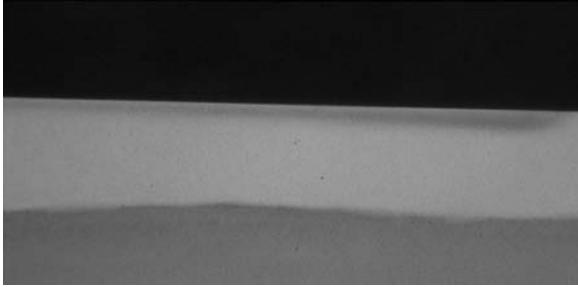
Fig. 9. Radiograph of 'wedge-shaped' enamel lesion. A dentine section was to the left during immersion in the acid-gel system. An increase in the severity of demineralisation can be observed moving left-right across the enamel.

the dentine used and in similar bovine enamel lesions were found to be of the same order, approximately 20 ppm, more was presumably released from the dentine because more of this tissue actually dissolved.

The remineralisation described above occurred in enamel immersed in gels adjusted to simulate plaque-fluid conditions, when next to dentine, presumably as a result of deposition of mineral originating mainly from dissolution of dentine. As there is no reason to suppose that similar precipitation could not occur in the dentine, it is likely that the rate of concurrent dissolution of sound dentine was greater than deposition of mineral within the dentine, and that the net effect was further demineralisation, whereas in the enamel the de-/remineralisation balance favoured net remineralisation.

Thus, it can be seen that where experimental conditions restrict the diffusion of dissolution products of enamel or dentine away from a developing lesion, these products can themselves modify the process. When substrates of differing composition are juxtaposed under these conditions, the interaction can cause pronounced effects on de- and remineralisation. These effects simulate well the conditions which might be expected in vivo at the EDJ and, in the case of the studies discussed here, may help explain the phenomenon of hidden caries.

3.6 Bovine Enamel as an Alternative to Human Enamel for the Study of De- and Remineralisation in vitro

Bovine enamel is a widely used alternative to human enamel for in vitro de- and remineralisation studies [55–57]. It is often more readily obtainable than human enamel, is more consistent in terms of chemical composition and relatively large, flat samples may be prepared, for example when plano-parallel samples are required for microhardness analyses. However, whilst extensive

reviews exist which cover the use of different types of hard tissue for intraoral studies [58, 59], comparative reviews for in vitro de- and remineralisation studies are relatively scarce. Here we consider the data available in the literature and compare the findings with those of our own, focusing on the qualitative and quantitative trends observed.

White and Nancollas [60] reported a K_{en} (the solubility product for enamel) value for bovine enamel of $5.9 \times 10^{-58} \, mol^9l^{-9}$, which matches exactly at least one commonly used literature value for pure crystalline hydroxyapatite [61]. Recently we estimated a value for K_{en} derived from empirical observations of bovine enamel lesion progression. Bovine enamel was immersed in solutions whose calcium, phosphate and buffer concentrations were similar to the '$DS_{En} = 0.36$' demineralising solution described by Zhang et al. [46], and pH was varied between 4.6 and 5.0. At pH 4.6 and 4.8, we found subsurface lesions, at pH 4.9 some superficial surface softening and at pH 5.0 no demineralisation was evident even after 56 days' continuous exposure. Allowing for enamel inhomogeneity and assuming that on average the solution was saturated with respect to bovine enamel at pH 4.9, we calculated a value for K_{en} of $1.7 \times 10^{-58} \, mol^9l^{-9}$ using a program developed by Larsen [62].

This value is higher than those cited earlier, possibly because of our use of relatively newly erupted enamel, which is less crystalline, and hence more soluble than enamel which has 'matured' [63]. However, it is very close to one value quoted for dissolution of bulk human enamel [64] and very close indeed to the value calculated by Zhang et al. [46], who suggested that demineralisation kinetics were controlled in part by a mineral phase with an apparent K_{en} of $2 \times 10^{-58} \, mol^9l^{-9}$. Given the divergence of reported values for both human and bovine enamel, it seems unlikely that comparison of K_{en} values alone is useful when comparing the behaviour of enamel types, except in cases where experimental conditions are very similar.

Lesions created in both bovine and human enamel, in an acidified methyl cellulose gel system, displayed many of the same qualitative trends [Lynch, unpubl. data]. After an initial period of approximately 3 days when dissolution was negligible, mineral loss was typically found at a series of discrete locations, with no apparent mineral loss between these 'pockets' of demineralisation. Surface zones were typically poorly defined or absent. After 5 or more days, the isolated pockets had coalesced and lesions were uniform in terms of both depth and mineral loss across the bulk of the lesion body, with well-defined surface zones. When observed under polarised light, these initial pockets of demineralisation were very often coincident with Hunter-Schreger banding. This was particularly noticeable in bovine enamel. Shellis [64] reported variations in solubility related to enamel microstructure and suggested that structure/solubility relationships are likely to influence lesion formation.

Anderson [65] monitored dissolution of human and bovine enamel in a flow-cell radiographically and noted qualitative similarities between the two enamel types. Both underwent an initial period of mineral loss which was sigmoidal with respect to time, followed by a protracted period where mineral loss proceeded in approximately direct proportion to time, with no clear distinction between human and bovine enamel. He suggested that the sigmoidal period might have been attributed to surface zone formation or some intrinsic property of surface enamel. The former effect may be related to our observation earlier, where initial pockets of demineralisation, without obvious surface zones, coalesced into well-defined lesions with frank surface zones. The enamel we used had been abraded and polished and so it was not, in this case at least, due to some property of the natural surface. However, whether or not enamel structure is involved, our observations are in agreement with those of Anderson in as much as we observe an initial nonlinear rate of mineral loss followed by the formation of a stable surface zone over a homogeneous lesion and relatively uniform progression thereafter.

After this initial non-uniform period, mineral loss proceeded as described by Arends and Christoffersen [66]. Typically the increase in mineral depth was best described by

$$l^p = \alpha t + q$$

where l is lesion depth, t is demineralisation period and α, q are constants. The value of 'p' was typically between 2 and 3 for both human and bovine enamel lesions in agreement with Arends' findings for lesions created in vitro, and suggests that in both cases lesion progression was predominantly diffusion controlled. With increasing time, 'p' tended towards 3 for both enamel types as the calcium and phosphate from the dissolving teeth slowed lesion progression.

Qualitatively, once again lesions were similar in human and bovine enamel with well-defined surface zones over distinct lesion bodies. Greater mineral loss occurred at the edges of lesions compared to the bulk of the lesion, and this was attributed to an effect similar to that described by Ruben et al. [52], where the dissolution products from the demineralising lesion inhibit progression of the lesion to a lesser extent at the edges because ionic diffusion is greater, and hence the elevation of DS_{En} is lower, as described in the previous section. The actual rate of mineral loss in bovine enamel was approximately twice that in human enamel in agreement with the findings of Edmunds et al. [67]. In their comprehensive comparison of enamel types, mineral loss in human, bovine, ovine and equine enamel was monitored quantitatively using polarised light. A model biofilm was used to simulate the effect of dental plaque. Qualitative trends were also studied using SEM. They concluded that the rate of mineral

loss in all animal enamel was roughly twice that in human enamel, and that qualitatively it was very different in ovine and equine enamel when studied using SEM.

Zhang et al. [46] studied the effect of demineralising solutions simulating plaque fluid on human enamel. Demineralising solutions with a range of DS_{En} values were made by the addition of calcium and phosphate ions to lactic acid buffer at approximately pH 5. Solutions were refreshed regularly to minimise the effects of dissolution products on lesion progression and mineral loss was quantified radiographically. Here, mineral loss was linear with respect to time, confirming Arends and Christoffersen's prediction [66]. Lynch [16] found very similar behaviour in bovine enamel, where again mineral loss was linear with respect to time. The 'R' parameter, as defined by Arends et al. [68], was typically 0.15–0.20, similar to values obtained in the same system for human enamel, but much lower than the value for lesions formed in acid-gel systems, which typically have an R parameter of 0.30–0.35, again regardless of enamel type. Thus, for both enamel types, the kind of demineralising system used can have a more pronounced effect on lesion characteristics than the intrinsic qualities of the enamel itself.

In aqueous systems which are initially infinitely undersaturated with respect to enamel, differences between human and bovine enamel can be very small. During enamel demineralisation studies, initial enamel dissolution rate was used to match samples prior to pH-cycling [Lynch, unpubl. data]. In this case, there was no significant difference between the two enamel types. Mean calcium demineralisation rates (SD, $n > 50$) for human and bovine enamel were 0.74 (0.26) and 0.66 (0.14) $\mu g \cdot mm^{-2} \cdot h^{-1}$ respectively.

Relative trends during de- and remineralisation pH-cycling studies are often similar in bovine and human enamel. In section 3.2 we discussed the effect of the physical size of lesions at baseline on subsequent mineralisation behaviour. Here, the observed effects were similar in both human and bovine enamel, both during pH-cycling and de- and remineralisation in isolation. Page [35] reported a dose-response of decreasing demineralisation of bovine enamel with increasing fluoride concentration when the fluoride was present in the experimental pH-cycling solutions continuously. Fluoride concentrations ranging from 0.014 to 0.2 ppm reduced demineralisation as effectively as fluoride concentrations in the range 10–1,000 ppm delivered intermittently from toothpaste. In very similar studies using human enamel, Lynch et al. [32] obtained results which were virtually identical in qualitative and quantitative terms. For example, fluoride, when continuously present at 0.014 ppm in all experimental solutions, reduced enamel dissolution by 19.1 and 19.9% in bovine and human

enamel, respectively. In addition, similar dose-responses for both enamel types have been established during many studies whose primary aim was to confirm the fluoride-bioequivalence of novel dentifrice formulations.

For fundamental studies of enamel structure, or where small differences in relative solubilities would be expected to be critical, there is probably no substitute for human enamel. Mellberg and Loertscher [69] found considerable differences in fluoride uptake to human and bovine enamel under acidic conditions and suggested that more rapid dissolution of bovine enamel was responsible. Marked differences in prism size [70], porosity [71] and orientation (e.g. decussation) [72], and in intersample homogeneity, between the two types of enamel may render bovine enamel less useful as a substitute for human enamel, when effects associated with enamel microstructure are being studied. Meurman and Frank [73] investigated erosion in human and bovine enamel. Whilst the surface ultrastructure of the eroded enamel was similar in both enamel types, variation in response within the human enamel samples appeared to be greater than variation between the enamel types. Structural heterogeneity within the human teeth was proposed as an explanation and, in this case, the more uniform response of bovine enamel caused the difference in behaviour. It would be interesting to extend this approach to try and determine which type of site, if any, within human teeth is intrinsically more caries-prone.

So, although bovine enamel is often a good analogue for human enamel when studying de- and remineralisation, careful thought should be given to experimental design in cases where, for example, differences in enamel microstructure or solubility might have a disproportionate influence. However, deliberately designing experiments to enhance differences in de- and remineralisation due to the contrasting prism structures in the two enamel types, should lead to a greater understanding of exactly how enamel microstructure influences caries progression.

3.7 Conclusions

We have discussed some of the many factors which should be considered when designing in vitro experiments to study enamel de- and remineralisation processes. Relatively minor modifications to the treatment regime and the substrate environment, and intrinsic differences in the substrate composition, can all have a profound effect on the outcome of such studies. However, these modifications and differences can be (a) used to increase the relevance of the studies to the corresponding in vivo situation or (b) deliberately exaggerated to gain useful information on, for example, the effect of enamel structure on caries.

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Tooth Wear

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4.1 Introduction

The term 'tooth wear' is commonly used to describe the loss of tooth hard tissue due to non-carious causes [1]. This encompasses a variety of both chemical and mechanical causes of both intrinsic and extrinsic origin. The term tooth wear is preferred over some of the more precise definitions of individual hard tissue loss mechanisms, because it acknowledges the fact that wear is usually a multifactorial process; one mechanism may dominate, but the overall wear is commonly due to the interaction between two or more wear mechanisms. In dentistry, the terms erosion, abrasion, attrition and abfraction are widely used to describe particular mechanisms of hard tissue loss.

This chapter contains a short review of factors affecting tooth wear, followed by a discussion of methods to measure tooth wear, illustrated with some selected examples. The chapter will not attempt to review the literature on tooth wear in detail, although specific publications of interest will be discussed. For detailed reviews, the reader is referred to papers by Azzopardi et al. [2] and Milosevic [3] and the book edited by Addy et al. [4].

4.2 Tooth Wear Mechanisms

The mechanisms of tooth wear fall into two distinct types: those of chemical origin (e.g. erosion) and those of physical origin (e.g. abrasion, attrition). In any individual, both chemical and physical insults to the tooth hard tissue will be present in some form or other, so tooth wear is the combined effect of these insults. Despite the clear definition of a number of distinct tooth wear mechanisms, it is uncommon to find a single wear mechanism present in the

mouth [1]. Thus, the term tooth wear is preferred over the individual mechanistic definitions, as it allows for the interactions between mechanisms to produce an overall loss of tooth hard tissue.

4.2.1 Erosion

Erosion is defined as the loss of hard tissue by chemical means not derived from bacteria, i.e. the dissolution of hard tissue by acid where the acid source is not the oral bacteria [5]. Erosion may be caused by either intrinsic (e.g. stomach acid) or extrinsic (e.g. dietary) sources. Erosion is often associated with the consumption of acid products, such as fruits or acid beverages, or with medical conditions where reflux of acidic into the oral cavity is present. Interestingly, the term erosion is widely used in other fields where the definition is rather different. For example, in the field of tribology, erosion refers to the loss of material from a surface by solid or liquid impacts [6]. In the classical tribological definitions, the mechanism dentists refer to as erosion would be described as corrosion, or tribo-chemical wear.

The loss of tooth hard tissue by erosion is due to the dissolution of the tooth structure under acidic conditions. Enamel is a hard, durable material, consisting primarily of hydroxyapatite crystallites that align to form a prism structure, and is sparingly soluble in water under neutral pH conditions. The supersaturation of saliva and gingival crevicular fluid with calcium and phosphate ions acts to prevent the dissolution of hydroxyapatite, and thus maintains the structural integrity of the tooth. However, under acidic conditions, the dissociation equilibrium shifts to favour the dissolution of hydroxyapatite. Thus, material loss from the enamel matrix occurs due to the dissolution of hydroxyapatite. This is the mechanism of material loss from enamel under both caries and dental erosion. Dentine and cementum are also affected by acid erosion. However, the collagen matrix in these materials helps to maintain some structural integrity as the hydroxyapatite is lost. In addition, dentine has some capacity to self-repair, where new sclerotic dentine is laid down in response to the material loss. Mild erosive lesions in enamel may also remineralise to some extent, but the body has no mechanism to repair significantly damaged enamel.

In the early stages of erosion, a marked softening of the demineralised enamel has been reported (see chapter 5). The enamel is softened to such an extent that it becomes extremely vulnerable to mechanical attack and virtually any mechanical action will cause additional loss of the softened layer.

4.2.2 Abrasion

Abrasion is defined as the mechanical removal of hard tissue by the repeated introduction of foreign bodies into the oral cavity that are in contact

with the teeth [5]. Typical causes of abrasion are consumption of abrasive foods, excessive or abusive use of oral hygiene products and occupational causes (e.g. workmen who hold screws or nails between their teeth).

Tooth enamel is rather resistant to mechanical attack, by virtue of its hardness and strength. Indeed, tooth enamel is said to be the hardest material in the human body. A high degree of hardness is required to resist the mechanical attacks which the teeth are subjected to on a daily basis, either through biting and chewing food, oral hygiene procedures, tooth–tooth contacts or even tongue–tooth contacts. Examination of the worn surfaces from extracted teeth reveals that the mechanism of wear from mechanical attack is primarily one of ductile plastic ploughing and grooving. In addition, there may also be an element of material loss by delamination, where subsurface cracks occur under repeated mechanical loading. If the cracks extend, material may be lost from the surface by chipping or delamination. In order for a material to be plastically deformed, it must be in contact with a second material, which has a hardness equal to, or greater than, the first material. If the second material is less hard than the first, then it will simply plastically deform itself and will not cause wear to the first material. Thus, abrasion of the tooth tissues will only occur when the tooth is in contact with a material which is harder than the enamel or dentine. Enamel has a high hardness (ca. 4–5 GPa, see chapter 5), which renders it resistant to mechanical attack by all but the hardest materials. By contrast, dentine is much softer (hardness ca. 0.5 GPa) and is, therefore, much more susceptible to abrasion.

The mechanism of material removal in abrasion involves a hard material (i.e. one which is harder than the surface being abraded), sliding over the tooth surface under load. The hard material causes the tooth surface to deform plastically and results in the formation of a groove. Some material may be pushed up to the sides of the groove to form pronounced 'shoulders'. Material loss occurs either from cutting or brittle cracking of the ploughed material, or through intersection of the grooves, where the 'shoulders' are removed.

4.2.3 Attrition

Attrition is the mechanical removal of hard tissue by direct contacts between teeth (either natural or restored) with no foreign substance intervening [5]. This mechanism causes wear by tooth–tooth contacts as well as by tooth–restoration, and indeed restoration–restoration contacts. The action of mastication and bruxism are known causes of attrition. In the field of tribology, the term abrasion refers to the loss of material from a surface by sliding, rubbing or scratching. Two-body abrasion refers to abrasion caused by two contacting surfaces in relative motion, i.e. the mechanism in dentistry that is described as attrition. Three-body abrasion refers to abrasion caused by surfaces in

relative motion where a hard abradant (the 'third body') is also present. Three-body abrasion is, therefore, consistent with the dental definition of abrasion.

From a mechanistic perspective, attrition is rather similar to abrasion, but in this case there are only two contacting materials, which may be of similar hardness (e.g. for tooth–tooth contacts) or of differing hardness (e.g. tooth–restoration contacts). Attrition is caused by contacts between natural or restored teeth during mastication or teeth grinding. The wear patterns observed are characterised either by scratching and grooving, where the asperities of one tooth surface contact another and produce ductile ploughing, or by chipping and delamination due to crack growth. This process may be accelerated where a tooth is restored using a hard material such as porcelain, and thus the ideal restorative material should be similar in mechanical properties to natural enamel.

4.2.4 Abfraction

Abfraction is a slightly more controversial tooth wear mechanism [7–8]. This is defined as the loss of hard tissue near to the cervical margin due to crack growth caused by the interaction of a number of mechanisms. The mechanical loading and unloading of the tooth during mastication causes tensile and compressive stresses to occur, leading to a point of stress concentration at the cervical margin. Through the cyclic loading and unloading, cracks are initiated in the cervical region and then propagate down into the tooth structure, perpendicular to the surface. As the cracks become elongated, material is lost by chipping, which produces a characteristic v-shaped notch lesion. In addition, there may be contributions due to erosion and/or abrasion, which further weaken the affected area and may accelerate the wear process, to produce complex abfractive lesions.

4.2.5 Interaction between Wear Mechanisms

In the complex environment of the mouth, many potential wear events occur everyday. The overall degree of tooth wear present is, therefore, related to the many wear events a tooth is subjected to over a lifetime. It is clear, as was mentioned previously, that tooth wear is a complex multifactorial process and, in the majority of individuals, wear will be due to the interaction of many different factors and mechanisms.

Of particular note is the importance of erosion in accelerating tooth wear. The softening of enamel and dentine surfaces by erosive attacks from acid renders the surface extremely susceptible to mechanical attacks [9]. Abfraction is also an example of interaction between wear mechanisms, where abrasion and erosion may act in combination with cyclic loading and unloading to produce an overall wear effect.

4.2.6 Protective Effects of Pellicle

The surfaces of the mouth are coated with a layer of salivary proteins known as the acquired pellicle, which provides a protective effect from both chemical and mechanical attacks to the tooth surface. The pellicle layer both moderates diffusion of ions away from the tooth surface, thus inhibiting the dissolution of enamel by erosion, and provides a lubricating layer to protect from mechanical attacks [10, 11]. It is known that dental prophylaxis and the use of regular oral hygiene procedures are able to reduce or remove the pellicle layer. However, recent evidence has shown that the pellicle is able to re-form very rapidly and thus, maintain a protective layer over the tooth surface [10]. For this reason, tooth wear studies performed in vivo or in situ, where the mediating effects of pellicle are present, will give a much more realistic assessment of tooth wear than in vitro studies. For a more detailed discussion on the effects of pellicle, see chapter 2.

4.3 Approaches to Assessing Tooth Wear

The multifactorial nature of tooth wear makes the study of this phenomenon difficult, and it is therefore not surprising that many of the reported studies focus on a single wear mechanism, often using laboratory models.

Virtually all dentifrice formulations contain abrasive particles, typically composed of amorphous silica, calcium carbonate, alumina or calcium phosphate. The mechanism by which the toothbrush and dentifrice interact to clean the teeth is one of abrasive cleaning. Toothpaste manufacturers thus aim to provide formulations with effective cleaning power, whilst minimising any wear to the underlying substrate. However, in such a system it is inevitable that some degree of abrasivity will be present, as toothpastes without abrasive particles are unable to prevent the build-up of extrinsic stain [12]. It is, therefore, important to understand any abrasion a dentifrice product may cause to the hard tissues in the mouth.

Tests in vitro have many advantages: they are relatively quick, simple, reproducible and offer a controlled environment. Test methods reported in the literature are primarily focused on a single wear mechanism and are often designed to investigate the performance of a particular type of product, such as toothpastes, denture cleansers and restorative materials. Wear tests relating to toothpastes are generally focused on abrasion, whereas tests relating to restorative materials often focus on attrition. Erosion and abfraction have received less attention in the literature, but a number of test methods have been reported. However, it should be recognised that wear tests in vitro are usually not designed to accurately mimic the complex interactions of the oral environment,

rather they are designed to discriminate between treatments of interest, and therefore the extrapolation of laboratory test results to clinical relevance should be used with caution.

In order to fully understand wear in the oral environment, in vivo clinical trials are required. These are necessarily complex studies, run over substantial periods of time to account for the fact that tooth wear is a rather slow process in all but the most extreme conditions. Additional confounding variables, such as diet, illness and environment, are difficult to avoid, which may reduce the power of any study to discriminate between test treatments. A further complication is the requirement to accurately measure minute changes in tooth surface profile on natural teeth. However, a number of studies have been reported and provide valuable information.

A third possible approach to measuring tooth wear is to use an in situ study design. Here, volunteers wear test specimens that are either inserted into a denture or appliance, or attached directly to the teeth. Following the test phase, the test specimens are removed and the wear is assessed using laboratory procedures. An advantage of this approach is that the test specimens are exposed to the multiple interactions of the oral environment, but the sensitivity of laboratory analysis is maintained. This increased sensitivity allows for much shorter duration studies compared to a full in vivo clinical trial and it is, therefore, possible to apply some control to variables such as diet and volunteer illness, if required.

4.3.1 Laboratory (in vitro) Test Methods

For laboratory tests to determine tooth wear, typically a test specimen is subjected to an exaggerated wear regime, involving contact with the abradant (e.g. dentifrice slurry or restorative material sample) or erodent (e.g. acidic beverage) of interest, and wear is measured after a specific number of loading cycles or after a defined contact time. The tests are often designed to maximise wear within a short period of time, and therefore discriminate between products, rather than accurately mimic the oral environment.

To examine dentifrice abrasivity, the most widely used methods are the radiotracer [13, 14] and profilometry methods [15, 16]. In the radiotracer technique [14], samples of either enamel or dentine from extracted human teeth are exposed to a radiation source. The radioactive samples are then brushed with a slurry of the test dentifrice in a brushing simulator under defined conditions of loading, slurry concentration and number of cycles. The abrasion is measured by assessing the radioactivity of the resulting slurry following the test, which therefore gives a measure of the amount of material abraded from the enamel or dentine surface. A standard reference abrasive (usually calcium pyrophosphate or calcium carbonate) is also tested using the same conditions and the abrasivity

of the test dentifrice is calculated relative to that of the reference material. This methodology, known as relative dentine abrasion (RDA) or relative enamel abrasion (REA), is widely adopted, and appears in both the British [17] and International [18] standards for toothpastes.

An alternative method involves using a stylus or optical profilometer [16]. Here, the enamel or dentine specimen is polished to give a smooth, flat surface. The profile is measured using a profilometer device and adhesive tape is applied to the specimen to leave an area of the tooth sample exposed. The sample is then brushed using a brushing simulator with a slurry of the test dentifrice under prescribed conditions. Following the brushing, the adhesive tape is removed and the surface profile of the specimen is remeasured with the profilometer. Using the area protected by the adhesive tape as a reference, it is possible to subtract the final surface profile from the initial profile, to calculate the cross-sectional area of the material removed.

Whilst the use of enamel and dentine as test substrates is widespread, they are complex materials to work with due to the natural variability both within and between specimens. A number of authors have examined alternative materials, which have similar mechanical properties to enamel and dentine, to use as test substrates. Acrylic [19, 20] and synthetic hydroxyapatite [21] have been proposed as suitable materials for abrasion testing, where mechanical effects dominate. These materials have several advantages since they are available as relatively large, smooth samples and exhibit better intra- and inter-sample reproducibility than their natural counterparts. This may, therefore, give better discrimination between test products for formulation development. However, the use of natural enamel and dentine is preferred, particularly for studies that aim to understand interactions between toothpaste products and tooth hard tissues. Other methods for assessing toothpaste abrasivity to hard tissues include gravimetry [22], scanning electron microscopy [23] and laser reflection [24].

For testing dental restorative materials, many regimes exist that use similar principles to those described for assessing toothpaste abrasivity. These tests may be conducted under conditions of two-body or three-body wear [25], i.e. focussing either on attrition or abrasion. Two-body tests for restorative materials either use human enamel [26] or a hard material, such as alumina [27] or steatite [28], as the abrader. For three-body tests, an abrasive medium, such as toothpaste slurry [29, 30], or an abrasive food, such as rice or millet seeds [31, 32], is typically used. These test methods are usually not truly representative of the oral environment; rather, they are designed to assess the wear resistance of restorative materials under extreme conditions.

In vitro tests to assess dental erosion have tended to concentrate on understanding the mechanisms of hard tissue removal, as these methods cannot

replicate the complexity of the oral environment. In a study reported by Davis and Winter [33], enamel and dentine specimens were exposed to a range of acidic solutions in vitro. The samples contained a region which was protected from erosion by PVC masking tape, and the tape was removed following the erosive challenge to allow measurement of the specimen profile using a stylus profilometer. The authors used solutions with a range of pH values to show that the erosion depth was related to pH in a logarithmic manner, with the most acidic solutions producing the most erosion. They found that for a 3-min exposure to acid at 25°C, the erosive loss on enamel, L (in microns), was related to the pH by the equation: $\log L = 2.2 - pH$.

4.3.2 Tooth Wear Clinical Studies (in vivo)

Clinical studies of tooth wear are complex long-term studies for reasons already described. Some researchers have attempted to make objective measures of tooth wear, often using replicate techniques, whilst others have employed tooth wear indices.

Early work by Saxton and Cowell [34] demonstrated the potential of replicate techniques, although they did not have the sophisticated measurement techniques that are available today. The study involved taking impressions of teeth with established cervical wear lesions and comparing impressions taken over two 6-month periods with positives cast from the impressions. The gap between the baseline impression and the 6-month positive was measured optically and this gave a measure of dentine loss. Using a crossover design, the authors were able to show differences in dentine wear between toothpastes with RDA values of 20 and 120. The differences in dentine wear, measured in vivo, were substantially smaller than those measured in the laboratory using the RDA methodology; 0.7 and 1.2 μm per week, respectively. Thus, whilst the laboratory abrasion (RDA) values differed by a factor of 6 for the two products tested, the difference in the dentine abrasion rate of the same products in vivo differed only by a factor of 1.7, which may be due to the moderating effects of the salivary pellicle.

The taking of tooth impressions to assess wear in vivo has become popular in recent years, as silicone impression materials are available with excellent detail reproduction and good dimensional stability. Several authors [35–38] have reported the use of these materials to capture the dimensions of selected teeth (or teeth surfaces) over a period of time. The silicone impressions are scanned using a profilometry system, typically a laser profilometer, to build an electronic image of the tooth surface. Several scans of the same tooth or surface, taken over a period of time, can then be electronically overlaid and subtracted, to calculate the change in surface contour, or the volume of material removed. This technique can only work if the two scans are precisely overlaid. To avoid

errors, the technique relies on sophisticated computer technology to overlay the two surface scans of interest. In a short-term study, the enamel may be assumed to be unchanged if little erosion is present and as such, may be used as a reference surface [36]. Other authors have cemented metal discs onto the tooth surface to act as a reference [37], although these are likely to display some wear in a long-term study.

Lambrechts et al. [35] described a method where silicone impressions of molar and premolar teeth were taken at 6-monthly intervals over a period of 48 months, in a study involving 21 subjects. The impressions were taken of teeth with established occlusal attrition and three reference points were ground into the enamel to form a common reference plane for measurements. The enamel wear was calculated by scanning positive replicas of the silicone impressions, using the reference points to calculate vertical wear. The authors found that the average vertical enamel wear over 4 years was 153 µm for molars and 83 µm for premolars. They also found that the wear rate showed a decreasing trend in each 6-month time period.

The use of tooth wear indices, such as described by Smith and Knight [1], is an alternative approach to studying tooth wear in vivo. Volpe et al. [39] describe the use of a tooth wear index to study wear in subjects using two different dentifrices over a period of 54 months. The authors reported that it was extremely difficult for the assessor to estimate the degree of tooth wear or to distinguish between erosion and abrasion. They, therefore, recorded for the presence or absence of tooth wear on each tooth and showed that the number of affected teeth rose over time, but there was no significant difference between the two dentifrices studied, despite the fact that they had very different laboratory abrasivity (RDA) values. The results of the study showed an average of 2.52 teeth with wear lesions for the lower abrasive dentifrice and 1.90 for the more abrasive dentifrice at the 54-month examination. The products had RDA values of 460 and 260; however, these were determined by assigning the reference abrasive (calcium pyrophosphate) an arbitrary value of 500. Using the current RDA methodology (where the reference abrasive is assigned a value of 100), the products would have RDA values of 92 and 52, respectively (see note[1]). A major drawback of this approach is that the slow progression of tooth wear requires a very long study (typically many years in duration) to show visible tooth wear. Hence, the instrumental methods using replication techniques are a valuable alternative.

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[1]All RDA and REA values quoted in this chapter were determined using the ISO recommended method [18], where a calcium pyrophosphate reference abrasive is used and arbitrarily assigned an RDA value of 100 and an REA value of 10.
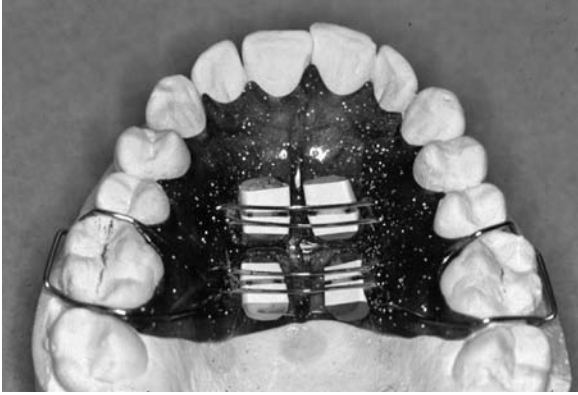
Fig. 1. Photograph of a typical palatal appliance containing four test specimens. Courtesy of N.X. West and M. Addy.

4.3.3 In situ Approaches to Tooth Wear

Assessing tooth wear using an in situ approach falls somewhere between in vitro and in vivo approaches. Essentially, this approach involves human volunteers wearing test specimens in the mouth for a period of time, during which they may be exposed to one or more test treatments. Ideally, an in situ methodology should expose the test specimens to the interactions of the oral environment, whilst maintaining the sensitive measurement techniques which may be applied to laboratory analysis.

Studies have reported success in using test specimens attached to dentures [40–42] or intraoral appliances [43–45], and, in a small number of examples, test specimens were cemented to natural teeth [46] or inserted into crowns or crown facings [47, 48].

In situ methods designed to study erosion have been described by a number of authors [43, 49–51]. West et al. [43] have widely reported an in situ methodology for assessing tooth erosion due to consumption of acidic beverages. Here, subjects wear enamel specimens in the mouth which are attached palatally to an upper removable appliance; a typical appliance is shown in figure 1. The subjects wear the appliances in the mouth during working hours and are supervised whilst drinking a pre-determined quantity of a test beverage. The test specimens are removed from the appliances at various stages in the study and the wear caused by erosion is assessed using a stylus profilometer. Figure 2 shows erosion values reported by West et al. [43] for enamel treated with orange juice and water in situ and clearly shows the erosive effects of an acidic beverage. The products were compared in a crossover design over two 15-day periods and the subjects consumed 1 litre of test beverage per day. It is
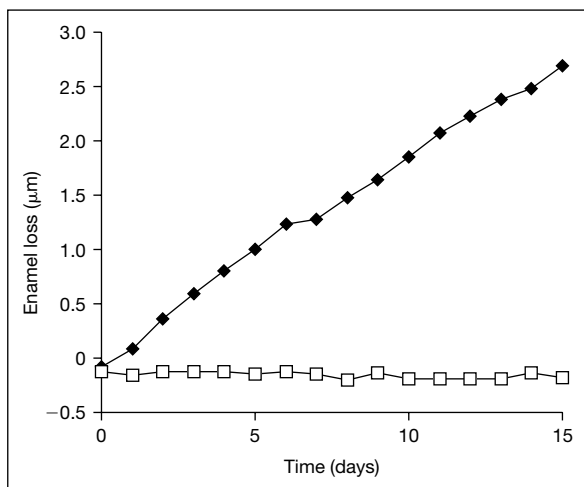
Fig. 2. Graph showing the loss of enamel in situ due to drinking orange juice (◆) or water (□), as reported by West et al. [42].

interesting to note that erosion is approximately linear over time in this model, with approximately 2.7 μm of enamel removed by drinking orange juice in the 15-day test period.

A modified version of this in situ methodology, using palatal appliances, has also been shown to be a potential means of assessing abrasion as well as erosion [52]. Addy et al. [52] reported a study where subjects wore appliances containing dentine specimens which were subjected to abrasion caused by toothbrushing. In a crossover design involving 10 subjects, they compared the abrasion caused by two toothpastes (A: RDA 85; B: RDA 189) over a 10-day period. The subjects wore the appliances during working hours and brushed the specimens with the test product five times per day for 60 s. Thus, the total exposure time to abrasion was $5 \times 60 \times 10 = 3,000$ s. Using the assumptions of Hooper et al. [53], this can, therefore, be considered to be equivalent to 300 days of natural brushing.

The influence of the salivary pellicle on erosion was demonstrated by Hannig and Balz [11]. Their work showed that in situ-formed salivary pellicle acts as a protective barrier against mild erosive challenges and can thus limit the damaging effects of erosion in the mouth. The data showed that the reduction in surface microhardness of enamel samples exposed to 0.1 and 1% citric acid solutions was significantly greater for enamel samples with no saliva exposure compared to enamel samples exposed to saliva for 24 h and 7 d in situ. Indeed, for short exposure times (30 s) to both acid concentrations, the pellicle-covered enamel blocks showed no significant softening compared to untreated enamel.

Fig. 3. Photograph of a typical denture containing one buccally mounted test specimen.

Other methods for assessing erosion in situ include transverse microradiography, where the remineralisation (or further demineralisation) of erosive lesions is studied on enamel blocks cemented to the lingual aspect of the lower incisors [46].

Several studies report the use of replica techniques to assess abrasion in situ [45, 48]. This methodology has also been successfully applied in vivo (see previous section). In this case, the wear is assessed using optical or interference microscopy. A drawback of this approach is that the production of replicas and positive copies inevitably provides a source of error and reduces the ability to measure small changes in tooth surface profile.

An alternative approach for assessing abrasion in situ has been described by Joiner et al. [40], in which enamel specimens were inserted into the upper buccal aspect of dentures; a typical example is shown in figure 3. The enamel specimens were polished flat prior to the in situ phase and indented using a Knoop diamond indenter. The lengths of the Knoop indents were measured optically and the subjects wore the dentures containing test specimens 24 h per day and brushed twice daily with a test toothpaste for 30 s. After 4 weeks of brushing, the specimens were removed and the Knoop indent lengths were remeasured optically. The amount of wear due to abrasion was then calculated from the geometry of the Knoop indentations. The authors reported a study in which the enamel wear of three different dentifrices (A: RDA 85, REA 3.4; B: RDA 189, REA 2.0; C: RDA 132, REA 42.7) was studied using this methodology, with a panel of 12 subjects in a factorial crossover design. Figure 4 shows the enamel wear and REA values obtained for the three dentifrices after 4 weeks in situ. Again using the assumptions of Hooper et al. [53], this is equivalent to 168 days
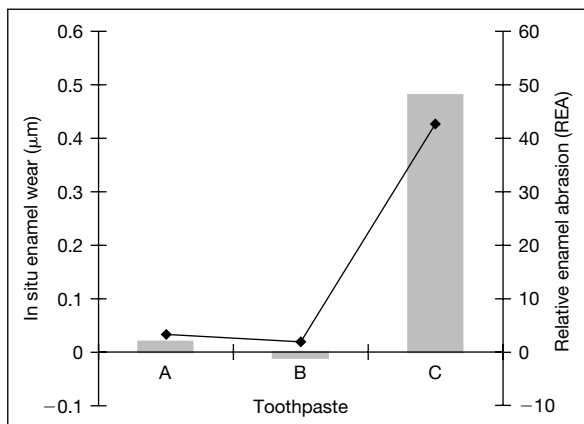
Fig. 4. Graph showing the loss of enamel in situ due to abrasion (bars) and relative enamel abrasion (REA, ◆) of three dentifrice products, as reported by Joiner et al. [40].

(24 weeks) of natural brushing. The authors clearly showed that the product with the highest REA value (dentifrice C) showed significantly more enamel wear in situ and in vitro than the other two test products. However, it was interesting to note that the lower REA products (dentifrices A and B) effectively showed no wear during the in situ study, despite the fact that they had measurable REA values. This is likely to be due to the protective effects of the salivary pellicle, which limit the abrasion of enamel in vivo. The authors have also published a variant on this method using enamel/dentine specimens [41], where the enamel wear was characterised using the indent methodology and the dentine wear was measured using an optical profilometer. Thus, in a single study, the authors were able to measure both enamel and dentine abrasion in situ.

The enamel/dentine in situ method was used in a study reported by Pickles et al. [54] to assess the abrasion caused by brushing with one of five different toothpastes covering a wide range of RDA and REA values. The results showed that for the conventional toothpaste products, the wear on both enamel and dentine was generally not significantly different, despite the fact that the RDA values differed by more than a factor of two. The results also showed that the wear rates for enamel and dentine were reduced as the study progressed, which is consistent with the observations of Lambrechts et al. [35].

A similar approach has been described by Murray et al. [42] using denture wearers, where indentations were placed within a small flat area which was ground on the buccal surface of the mesial cusp of the lower right first molar tooth of each denture. The subjects brushed twice daily with a test product and assessments were made at 1 and 6 months. The assessments involved examining

the visibility of two Vickers indents of different sizes, and the authors concluded that this methodology was able to discriminate between a range of toothpastes and denture-cleaning pastes.

In situ models have also been shown to be valuable in understanding the interactions between wear mechanisms in the mouth. In a recent study, the interplay between erosion and abrasion was examined using an in situ methodology [53]. Fifteen subjects wore both enamel and dentine specimens in palatal appliances and these were subjected to both erosion (via drinking orange juice or water) and abrasion (via brushing with one of two different test toothpastes) in a multiple crossover design. The wear was measured using a stylus profilometer, after 5 and 10 days treatment on each test regime. The authors showed that erosion and abrasion can act in combination, and the synergistic effect between the two mechanisms approached statistical significance for enamel ($p = 0.06$). They also showed that there were no significant differences between the abrasion or erosion/abrasion produced on enamel when two different toothpastes (A: RDA 189, REA 2.0; B: RDA 85, REA 3.4) were used. It should be noted that whilst the orange juice consumption in this study was typical of reported usage patterns, the toothbrushing events were exaggerated compared to typical reported usage. Examining synergy between erosion and abrasion, using this model, should therefore be approached with caution. Other studies in vitro have similarly shown that erosion and abrasion may act synergistically [9, 33]. However, exposure to saliva and fluoride promotes remineralisation of these lesions and may provide protection against further wear [55–57].

Jaeggi and Lussi [57] examined erosive lesions produced in vitro which were allowed to remineralise in situ using intraoral appliances and then brushed with a dentifrice product. The abrasion wear was measured using an indent length model similar to that used by Joiner et al. [40]. The authors reported that the enamel samples showed significantly less abrasion when allowed 60 min remineralisation time in the mouth compared with brushing immediately after the erosive challenge.

Eisenburger et al. [9] have examined the interplay between erosion and abrasion in vitro, and studied the effects of alternating erosive and abrasive challenges compared with simultaneous erosion and abrasion. The authors showed that simultaneous erosion and abrasion produced more wear to enamel samples than alternating the two challenges separately. In everyday life, the mouth is subjected to erosive and abrasive challenges at various times. In many studies, an erosive challenge is followed by an abrasive challenge, to simulate a typical situation where individuals may consume an acid food or beverage and then brush their teeth. However, the converse may be true for individuals who brush their teeth before eating, and thus it is important to understand the order effects when multiple wear challenges are present. The

situation where erosion and abrasion are present simultaneously would be consistent with consuming foods which are both acidic and abrasive or fibrous, for example, apples.

4.4 Implications for Oral Hygiene Products

In order to fully understand the implications of oral hygiene procedures on tooth wear, it is necessary to employ a number of the approaches described in this chapter. Whilst laboratory techniques offer high sensitivity and good discrimination between test products, the results from these studies cannot easily be translated into clinical significance. Clinical trials are also valuable, but it is important to recognise that there may be confounding factors, particularly in long-term studies, unless very strict controls are applied. Subject compliance may also be an issue in long-term studies. In situ studies would seem to offer an excellent balance between the sensitivity of laboratory analysis and the complex environment in the mouth. Whilst care should be taken to ensure that any study protocol provides a realistic approximation of the in vivo situation, there is considerable scope to further understand the phenomenon of tooth wear using in situ approaches.

Hooper et al. [53] have recently published a possible approach for extrapolating the results of an in situ study to a lifetime of brushing. This involves making several assumptions about the duration of a typical toothbrushing event and the amount of abrasion that may be considered clinically acceptable, but this does allow the estimation of the overall degree of abrasion, which would be produced by using a particular product for a significant period of time.

It is clear from the huge body of reported data in the literature that tooth enamel is very resistant to mechanical wear by abrasion or attrition, and these mechanisms only become important in cases of abusive or excessive product use. Enamel is also rather resistant to erosion and only becomes a clinical issue in cases of over-exposure to erosive products or of gastric reflux. Dentine is less resistant to abrasion and erosion, but evidence from recent in vivo and in situ studies suggests that brushing with a toothbrush and toothpaste produces limited wear to exposed dentine in a lifetime of use and will only reach clinically significant levels in cases of abuse. Modern oral hygiene products provide an excellent balance between maximising cleaning efficacy whilst minimising hard and soft tissue degradation.

Recent evidence of the structure of enamel exposed to an acid environment shows the fragile structures which remain in the softened surface zone following an erosive challenge [58]. Ganss et al. [59] also showed that when enamel specimens were brushed in vitro immediately after an erosive challenge, the

brushing force has no effect on the overall material loss. When considered with other publications discussed in this chapter, this would seem to point to a residual surface zone which is easily removed by virtually any mechanical action, so the role of the toothpaste in the interaction between erosion and abrasion is minor, and the abrasivity of the toothpaste is not a factor because the softened enamel is vulnerable to any mechanical attack.

It should also be recognised that brushing with a toothbrush and toothpaste has significant beneficial effects. In particular, several authors have reported that the presence of a fluoride-rich layer close to the surface of the tooth enamel or dentine has a significant protective effect against erosive and abrasive challenges [60–62].

4.5 Summary/Conclusions

Tooth wear is a complex phenomenon with a multifactorial aetiology. Many approaches to studying tooth wear have been proposed, ranging from simple in vitro models assessing a single mechanism to more elaborate in vivo and in situ studies where multiple interactions are present. Each approach presents different advantages and disadvantages. Clinical investigation of tooth wear (in vivo) provides the most realistic evidence, but has disadvantages in the duration of time required for a study to show significant effects and in the difficulties in controlling confounding variables. Laboratory studies (in vitro) generally allow a high degree of control, but are often considerably simplified and do not account for all of the interactions within the mouth. In situ studies offer a compromise between these two approaches and are therefore very valuable. Ideally an in situ study should deliver meaningful results within a short time period (typically a few days or weeks), allow the use of sensitive measurement techniques in the laboratory phase and also allow samples to be exposed to the multiple interactions in the mouth, whilst controlling other variables as necessary.

From the data presented in this chapter, it is clear that erosion, abrasion and attrition are all able to cause wear of enamel and dentine under the right conditions and may interact to some degree. Some evidence exists that these mechanisms may act synergistically, although more research is required to confirm this. There is also a significant body of evidence which suggests that, for most individuals, tooth wear should not reach clinically significant levels within a lifetime of product use. However, abuse or overuse of oral hygiene procedures or excessive consumption of acidic foods or beverages may lead to significant tooth wear, which will be detrimental to the individual from either an aesthetic or functional perspective and may require professional intervention.

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Nanomechanics, Chemistry and Structure at the Enamel Surface

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5.1 Introduction

Enamel is the hardest biological substance in the human body and is a composite material consisting of both a mineral and an organic phase. The mineral phase predominates (95–96 wt.%) and consists primarily of calcium phosphate salts in the form of nanoscale hexagonal hydroxyapatite crystals that are both carbonated and defective. Sets of similarly orientated crystals form rod-like structures called enamel prisms, 3–6 μm in cross-sectional diameter. Prisms are separated from each other by a thin organic prism sheath and by interprismatic enamel. The protein/organic matrix comprises approximately 1 weight percentage (wt.%) of the enamel, and the remaining approximately 3% is contributed by water [1]. The complex microstructure of enamel leads to large variations in mechanical behavior. These are particularly pronounced when comparing the mechanical properties of the occlusal surface, where the elastic modulus $E > 100$ GPa and the hardness $H > 5$ GPa, to those close to the enamel-dentine junction (EDJ), $E < 60$ GPa and $H < 3$ GPa.

The behavior of the enamel surface is particularly important in understanding the early stages of dental caries (cavities). Caries develop from demineralized regions of enamel commonly called either 'white spot' or 'carious' lesions. The lesions develop as a result of acids attacking the inorganic, apatite phase of the enamel. The acid is a by-product from acidogenic bacteria that are anaerobically consuming carbohydrates on the enamel surface. During the development of a lesion the above mechanical properties of the enamel show a dramatic fall in the demineralized region. Within a mature lesion the hardness and elastic modulus may be just a few percent of their normal values. There is often, however, a relatively narrow, remineralized region close to the surface of the lesion that is noticeably stronger than the lesion's interior. Chemical profiles

across the depth of a lesion indicate that there is a strong correlation between the lesion's mechanical behavior and its degree of mineralization.

In the oral cavity the surface of dental enamel is covered by a thin layer of salivary pellicle. If the dental enamel is cleaned with, for instance, an abrasive dentrifice, a pellicle layer begins developing within a matter of minutes. As it matures, it reaches a thickness that may exceed a micron. Eventually, micro-organisms and carbohydrates are incorporated into the layer resulting in a microbial film known as dental plaque. The microorganisms may include the acidogenic bacteria associated with the development of caries. The mechanics and structure of the pellicle are influenced by dietary elements such as tannins. Oral care products are frequently aimed at removing, bleaching or otherwise modifying the pellicle layer, typically to reduce the risk of dental disease or to improve the aesthetic appearance of the teeth. However, the pellicle layer is also believed to be important as a chemical barrier to acidic attack of the enamel sur-face and as an organic lubricant during mastication. Knowledge of the pellicle's mechanical behavior and its dependence on diet is, therefore, important in understanding the role of pellicle in preventing wear and carious lesions. Understanding the effects of abrasives and chemicals on the pellicle is impor-tant in the development of new oral hygiene products which reduce the risk of dental disease and improve aesthetic appearance without impeding the function of pellicle as a protective layer. For further information on the pellicle, caries lesions and tooth wear see previous chapters 2, 3 and 4, respectively.

This chapter describes the results of an ongoing study we are conducting into the nanoscale mechanical properties, chemical composition and structure of healthy enamel, carious lesions and the acquired salivary pellicle layer. A variety of material characterization techniques are being used, including: nanoindentation, scanning electron microscopy (SEM), electron microprobe analysis (EMPA), scanning acoustic microscopy, atomic force microscopy (AFM) and time-of-flight secondary ion mass spectroscopy (TOF SIMS).

5.2 Healthy Molar Enamel

5.2.1 Mechanical and Chemical Characterization

Enamel has often been viewed as a homogeneous solid [2, 3], but Knoop microhardness tests [4, 5] and compression tests [6] have shown that the Young's modulus (E) and hardness (H) are higher for cusp (or surface) enamel than for side (or subsurface) enamel. Depth-sensing Vickers indentation [7] has shown that the H and E obtained from an occlusal section of enamel are higher than those for an axial section. The variations in mechanical properties with location have been explained in terms of the degree of tissue mineralization. Notably,

microhardness and mineral content have been found to correlate in human enamel [8]. Similarly, it has been estimated that E is capable of changing by 3 GPa for each 1 vol.% change in mineralized hydroxyapatite [9]. Finite-element modeling of enamel using the volumetric crystalline fraction of hydroxyapatite [10] found that E increases from 93 to 113 GPa parallel to the crystal direction as the crystalline fraction increases from 0.81 to 0.99, but perpendicular to the crystal direction the variation is from 19 to 91 GPa for the same crystalline fractions.

In examining the mechanical properties of enamel, it is important to remember that hydroxyapatite is known to be a mechanically anisotropic material. Spherical-indentation testing of enamel has indicated that differences in mechanical properties may be related to prism orientation. Staines et al. [9] found that enamel hardness was higher when the indentation direction was parallel to the basal plane of the hexagonal hydroxyapatite crystals and lower when the indentation direction was normal to the basal plane. In a similar manner, Rasmussen et al. [11] found that enamel's work of fracture is very anisotropic, being lowest parallel to the prisms. Xu et al. [7] reported that fracture toughness varies by a factor of three, depending on prism alignment and crack orientation.

To further our understanding of enamel's mechanical behavior, nanoindentation has been used to examine local variations. Using nanoindentation the mechanical properties of very small, submicrometer volumes can be measured with fine spatial resolution. Performing thousands of individual indentations on an enamel sample enables the variations in mechanical properties across the sample to be plotted. Figure 1 shows the variations in E and H across an upper 2nd molar [12]. The data were obtained with a Nanoindenter XP™ (MTS, Knoxville, Tenn). This instrument has a lateral-positioning resolution of 1 μm and can apply loads of just a few microNewton while simultaneously measuring displacement changes <1 nm. Traditional methods for determining H only take into account permanent (plastic) deformation and require the visualization and measurement of indents [4, 5]. Nanoindentation, however, accounts for both plastic and elastic deformation, as well as time-dependent effects. In addition, it removes the requirement of indent visualization because both depth and applied load are continuously monitored throughout the indentation process [13]. Nanoindentation has been utilized on several occasions for the examination of dental tissue. Kinney et al. [14] used it to show that the H and E of intertubular dentine decrease with distance from the EDJ. Similarly, van Meerbeek et al. [15] used nanoindentation to characterize the H and elasticity of the resin-dentine bonding area. For enamel, however, nanoindentation has generally been used to determined 'average' values of H and E that are not site-specific: 3.39 ± 0.18 and 90.59 ± 16.13 GPa, respectively [3]. Figure 1 shows there are substantially larger variations in E and H than has normally been reported for enamel.
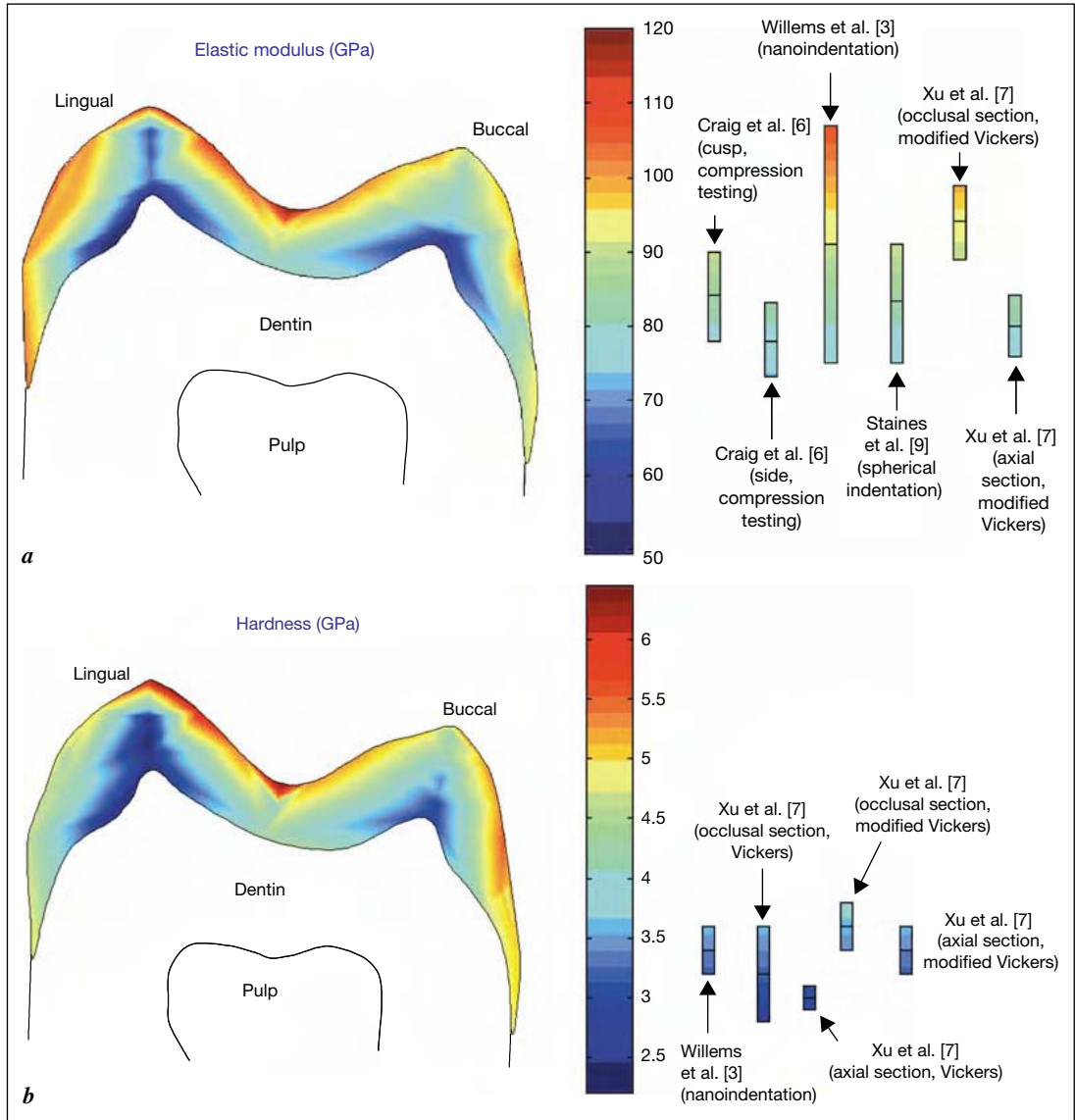
Fig. 1. Young's modulus, *E*, (*a*) and hardness, *H*, (*b*) for the enamel of the mesial half of a maxillary 2nd molar as determined by nanoindentation. The standard deviations for these averages range from 0.2 to 0.3 GPa for hardness and from 2 to 5 GPa for modulus. Note the wide variation in mechanical properties between the enamel surface and the EDJ. Average values of *H* and *E* that have been reported earlier by other researchers are included for comparison.
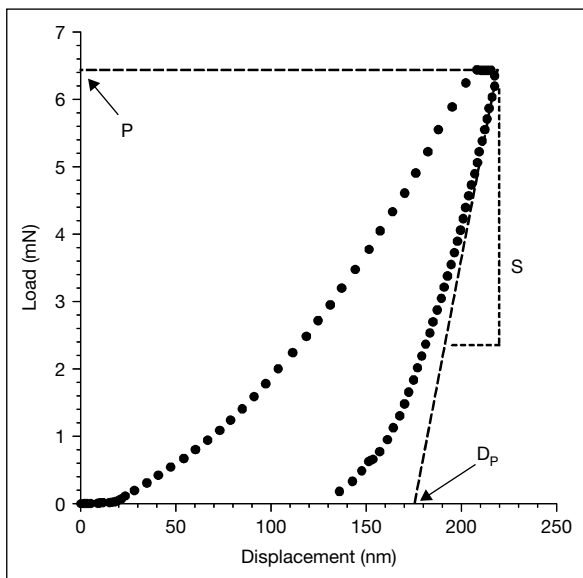
Fig. 2. Typical load vs. displacement curve for a nanoindentation in tooth enamel. The contact depth (D_p), area and stiffness (S) were all determined at the maximum load (P) by fitting a polynomial expression to the upper 70% of the unloading curve, and they were used to quantify hardness (*H*) and Young's modulus (*E*) for each nanoindentation according to standard routines [13].

The data for figure 1 were obtained by performing over 2,000 nanoindentations on the enamel of a cross-sectioned tooth (lingual to buccal section). A typical load-displacement curve for the enamel is shown in figure 2. The nanoindentation data were analyzed using standard routines [13] to provide *H* and *E* for each nanoindentation. This analysis requires a value for Poisson's ratio to be used: it was assumed to be 0.25. The tooth was embedded in a polymer mounting block, sectioned with a diamond saw and mechanically polished to give an optically flat finish. Note that ultrasonic cleaning was avoided during the sample preparation as it tends to crack the sample and loosen its mounting. One half of the sectioned tooth was used for mechanical characterization and the other half for chemical and structural characterization.

For all mineralized tissues, the environment in which they are tested can significantly affect their mechanical properties. For bone, tests in aqueous and in simulated physiological solutions can change the hardness and elastic modulus by 20% [16, 17]. For enamel and dentin, the difference between the 'dry' and 'wet' mechanical properties can be 10% [18, 19]. Earlier studies [9] found

that E increased by approximately 15% when a wet sample was allowed to dry in air for over 72 h. Similar changes are seen in H. However, this enhancement is expected to be fairly uniform over an entire enamel sample and, therefore, not to affect the relative values and trends within a single sample of enamel. When comparing the properties of different teeth or performing experiments that take many hours, it is necessary to make sure the teeth are in the same state of hydration. In an ambient laboratory environment, the absolute values of the measured elastic moduli are expected to be slightly higher than they would be if the samples were tested in a wet environment, but the environmental effects are minimal if the tooth has been stored correctly (a humid, but not wet, environment). Note that a wet environment is likely to give values closer to those found in vivo.

From a materials perspective there are two possible reasons why dental enamel shows the large variations in mechanical properties shown in figure 1: firstly, chemical variations in apatite composition and, secondly, changes in enamel structure with position from the occlusal surface to the EDJ. The chemical composition of enamel can be examined with a lateral resolution of 1–10 μm with electron microprobe analysis. Enamel structure can be obtained with SEM. To perform an accurate microprobe analysis, natural and synthetic minerals are used as standards to calibrate the instrument. This is fairly routine for geologists and earth scientists who are able to obtain chemical compositions with an accuracy of <0.1% for a wide range of elements simultaneously (including Na, Mg, Al, Si, P, K, Ca, Ti, Cr, Mn, Fe, Y, Zr, Ba, La, Ce, Pr, Nd, Sm, Gd, Dy, Er, Yb, Hf, Ta, Pb, Th, U, F and Cl). In enamel only a few of these (Na, Mg, Al, P, K, Ca, Ti, Cl and F) are above the detection limit. The Ti is likely to be an impurity or contaminant rather than a constituent of enamel. This technique does not work for lighter elements such as C, S, O and N which may be present in enamel.

The maps of mechanical properties (fig. 1a, b) show that H and E decrease on average from 4.6 to 3.4 GPa (a 26% decrease) and from 91.1 to 66.2 GPa (a 27% decrease), respectively, on going from the enamel surface to the EDJ. The extreme variation, however, from the maximum H of 6.4 GPa to the minimum H of 2.7 GPa is well over 50%. Equally large variations were observed in E (120 to 47 GPa). Note also in figure 1a and b that there is a significant drop in H (>50%) and E (>50%) on moving from the enamel surface to the EDJ on both the buccal and lingual sides of the molar. Note also that the values of H and E for the interior of the lingual side of the molar are generally lower than those found in the interior of the buccal side. The maximum H (>6 GPa) and E (>110 GPa), however, appeared at the lingual cusp and extended along its occlusal surface to the center of the molar. In contrast, the occlusal surface of the buccal cusp had a relatively low H (4.6 GPa) and E (93 GPa).
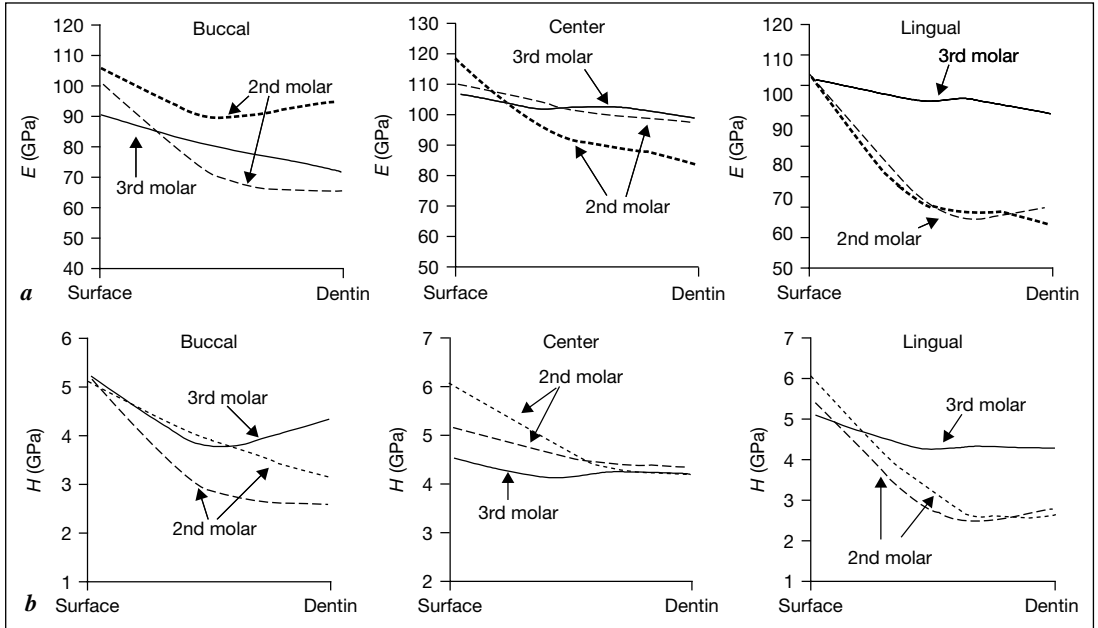
Fig. 3. Comparison between the mechanical properties of different teeth. The Young's modulus (*a*) and the hardness (*b*) for two different maxillary 2nd molars and a 3rd molar.

The trends in *H* and *E* observed in the tooth of figure 1 were found by Cuy et al. [12] to be similar in other upper 2nd molars, as shown in figure 3. However, it was reported that upper 3rd molars had some mechanical similarities, but were distinctly different in other ways to upper 2nd molars (fig. 3). Comparing the 2nd and 3rd molars, the trends in *H* and *E* suggest that these teeth have similar surface properties but different interior properties. The interior regions below the buccal and lingual cusps of 3rd molars were found to be generally harder and stiffer than the corresponding regions in 2nd molars, particularly for the lingual cusp (fig. 3).

Compositional variations in the distal half of the 2nd molar of figure 1 were found to follow some distinct trends. These are shown in figure 4. Earlier studies have shown that the major inorganic compounds of human tooth enamel are calcium (Ca), phosphate (P), carbonate, magnesium and sodium [20]. In the study by Cuy et al. [12], the wt.% of P_2O_5 and CaO decreased on moving from the outer rim of the enamel towards the dentine. This was true for all three traverses: center, buccal and lingual. MgO, Na_2O and K_2O displayed the opposite
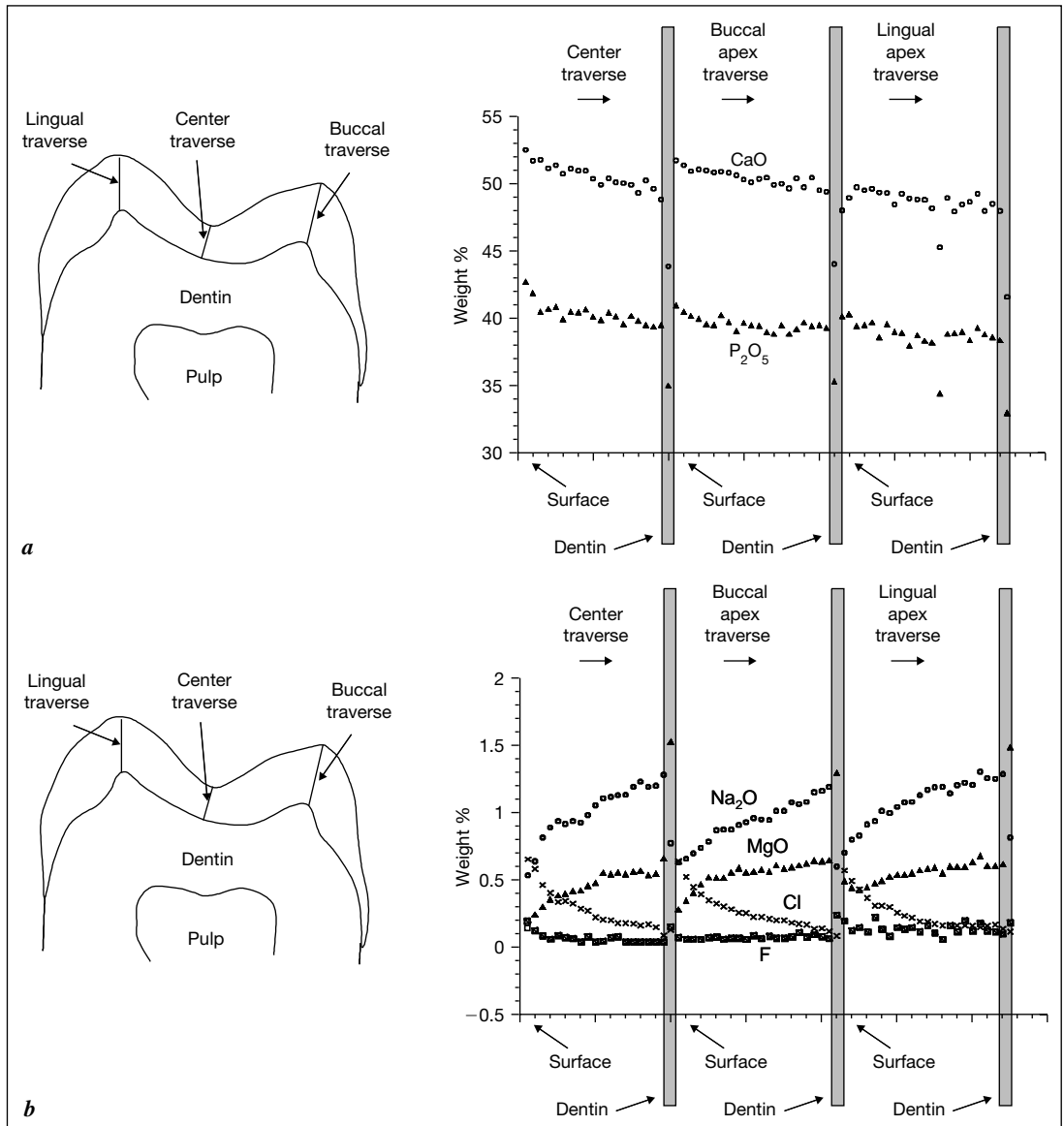
Fig. 4. Results of the electron microprobe chemical analysis of the distal half of the molar shown in figure 1. The values are weight percentage as a function of location. The plots are each broken into three sections for the three different traverses shown in the schematic. Points on the left side of each section represent values at the enamel surface; points on the right side correspond to interior values near the EDJ. The single points bordered by solid lines on both sides represent values found in the dentine.
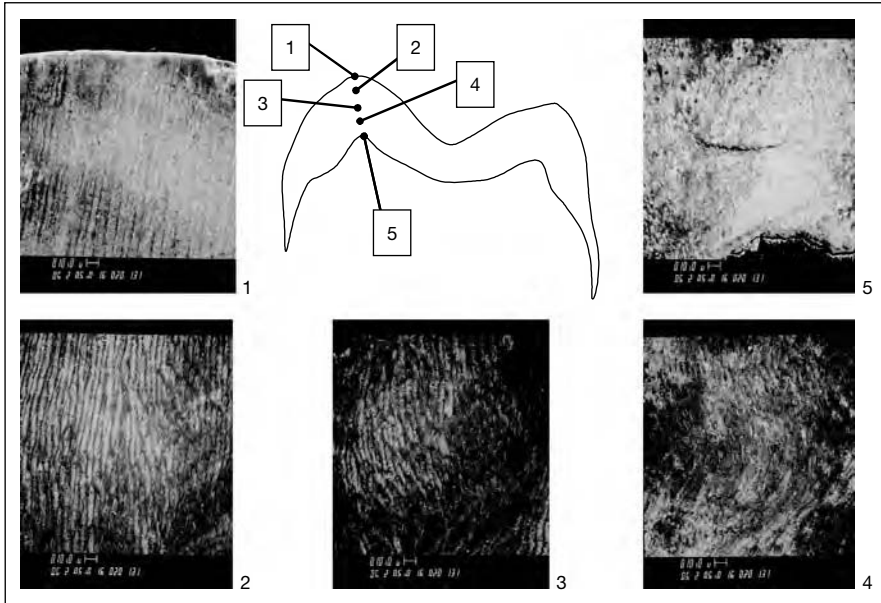
Fig. 5. Lingual traverse: SEM images of the axial section surface of a maxillary 2nd molar. Scale bar = 10 μm.

trend, with significant increases on moving towards the EDJ. Fluorine content showed no significant statistical trend, although the center traverse showed a slightly higher fluorine content at the enamel surface. Dramatic chemistry changes at the EDJ are also seen. Going from enamel to dentin: MgO showed a 134% increase (averaged over the three traverses) and Na_2O, CaO and P_2O_5 underwent 43, 11 and 12% decreases, respectively. The fluorine content was found to increase by over 50% in each traverse.

The enamel microstructure (fig. 5), as seen in a SEM after being etched for 15 min with an aqueous 0.005 M citric acid solution, showed large changes from the lingual cusp to the EDJ. Figure 6 shows the change in structure from the buccal cusp to the EDJ. At the lingual cusp the enamel prisms are normal to the occlusal surface (fig. 5), but their orientation is less well-defined in the interior of the enamel. The prisms lie in a number of different directions close to the EDJ, with many of the prisms at angles <90° to the occlusal surface. The prisms at the buccal cusp are mostly normal to the occlusal surface, but not as clearly ordered as the prisms at the lingual cusp. The interior prisms on the buccal side are generally not normal to the surface, and this is most obvious at the EDJ.
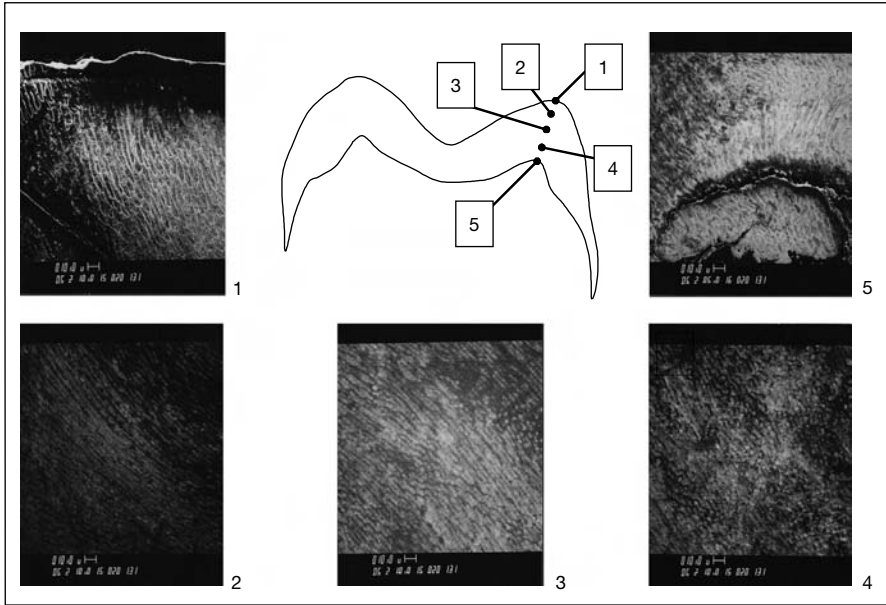
Fig. 6. Buccal traverse: SEM images of the axial section surface of the same maxillary 2nd molar as figure 5. Scale bar = 10 μm.

5.2.2 Comparison with Previous Studies

The trends in mechanical properties with position in the enamel (fig. 1) are similar to those reported previously for the hardness of enamel, though the magnitude of the variations is more pronounced when nanoindentation is used. Using Knoop indentation, Willems et al. [3] reported a 10% decrease in Knoop hardness from 287 for surface enamel to 259 for enamel at the EDJ. Knoop indentation in that case, however, was initiated some 300 μm from the enamel surface and only extended to 1,500 μm below the surface. Thus, the large variations observed with nanoindentation would have been missed. With nanoindentation, the highest values for H and E were found to lie within 100 μm of the occlusal surface and the lowest are in specific regions close to the EDJ. In addition, the Knoop indentation tests by Willems et al. [3] could not provide a dependable measure of E, and thus trends in this property were not established. Nanoindentation also highlights variations in H and E between the buccal and lingual sides of the tooth. In general, both H and E for an upper 2nd molar are higher along the surface of the lingual cusp than along the buccal cusp. In contrast, the interior of the lingual cusp appeared to have a lower H and E than the interior of the buccal cusp.

Figure 1 also includes previously reported values for H and E for comparison with the mechanical property maps of Cuy et al. [12]. It is clear that nanoindentation is able to sense a much wider variation in these properties than previously observed. This is at least partially because the past investigations of mechanical properties yielded mainly average values of H and E that correspond to the values for the interior enamel. Many of these previous studies did not show the extreme local variations that can be measured with nanoindentation. Only the earlier nanoindentation studies have shown any evidence for the highest E and H found by Cuy et al. [12] using nanoindentation. Willems et al. [3] reported $E = 90.59 \pm 16.13$ GPa and Mahoney et al. [21] reported $H = 4.88 \pm 0.35$ GPa.

Over the last 25 years, many researchers have characterized the chemistry of human enamel as a function of position [22–27], and in some cases the results have been correlated with spatial variations in its mechanical properties [8–10, 25]. As shown by figure 4, the ranges of Na_2O, MgO, Cl and F concentrations are similar for the center, buccal and lingual traverses, suggesting that variations in these elements do not explain the major differences in H and E that are observed across the three regions of the enamel. The measurements of CaO and P_2O_5, which correspond directly to changes in the degree of mineralization, more closely correlate with changes in mechanical properties. That is, mineralization (CaO and P_2O_5 concentration) is highest at the enamel surface of the center traverse, and this location also displays the highest H and E of the entire molar. The traverse with the lowest CaO or P_2O_5 was the lingual cusp, and this interior region also showed the lowest H and E. The relationship between mechanical properties and degree of mineralization has been widely reported [9, 10, 25]. In addition, reductions in mineralization near the EDJ have also been associated with increased porosity and increased water content [28], which could also act to reduce H and E.

In addition to the mesoscale variations in chemistry, it is also interesting to consider local variations in chemistry that result from microstructural features such as rods, tufts, lamellae and spindles [29]. Enamel tufts, lamellae and spindles contain larger quantities of organic material and are lower in total CaO/P_2O_5 wt.% than surrounding, highly mineralized enamel rods. These structures are also correlated with specific regions within the enamel. Tufts fan out from anchor points near the EDJ, lamellae tend to be 'cracks' running the entire width of the enamel, and spindles have been observed at the cuspal summits. These general observations compare favorably with the trends for CaO and P_2O_5 (fig. 4). For example, the lower wt.% of CaO and P_2O_5 near the EDJ could be attributed to the presence of hypomineralized tufts near the dentine. Similarly, the isolated fall in Ca/P shown in the lingual-cusp traverse (fig. 4) could be attributable to the presence of lamellae. Nanoindentation data on

enamel occasionally contain a single nanoindentation with very low E and H. These values may correspond to nanoindentations being performed in enamel tufts, lamellae or spindles. To confirm this would require chemical and structural analyses to be performed in the exact spot where the nanoindentation was made, which has yet to be done.

As well as compositional changes, the mechanical anisotropy of hydroxyapatite means that the influence of prism orientation on the mechanical properties of enamel must be considered. SEM images (fig. 5, 6) show significant changes in prism alignment with position within the enamel. Near the occlusal surface the long axes of prisms are aligned perpendicular to the surface. Near the EDJ, the long axes of the prisms are more randomly oriented, but generally aligned parallel to the occlusal surface. With respect to the surface used for nanoindentation testing, at the EDJ the prisms are both perpendicular and parallel to the test surface, but at the occlusal surface the prisms only lie perpendicular to the indentation direction. The alignment of the prisms relative to the surface used for nanoindentation testing means there is a significant change in the loading geometry of the prisms from the occlusal surface to the EDJ. Spears [10] modelled the effects of loading geometry on enamel prisms and found that the values of H and E were lower at the enamel surface, where the prisms experience just transverse loading, than at the EDJ, where the prisms experience both parallel and transverse loading. However, the opposite trend is observed in the nanoindentation data of figure 1: H decreases on average by 26% on going from the enamel surface to the dentine, and E decreases on average by 27%. The extreme variations are much larger. Thus the prism alignment or loading direction cannot be solely responsible for producing the observed trends in H and E. While the reported variations in mechanical properties do not follow the predictions based on the loading geometry of prisms, they do agree with some earlier work. Staines et al. [9] reported that the E of side enamel was approximately 24% greater for areas in which indentation was performed transverse to the prisms (as at the occlusal surface) than for areas in which the indentation was conducted transverse and parallel to the prisms (as at the edge of the dentine). It may be that enamel chemistry is the controlling factor, as suggested by Staines et al. [9].

The structural and chemical data, figures 4–6, indicate a higher percentage of presumably organic/hypomineralized material closer to the EDJ than at the occlusal surface. This lower degree of mineralization in the interior layers of enamel is expected to make it softer and more compliant. This may be true regardless of indenter/prism loading geometry. However, given the complex decussation of prisms in human enamel, more precise monitoring of prism orientation, microstructure and degree of mineralization is needed to evaluate

definitively the relative influence of mineralization (chemistry) and prism orientation on the mechanical properties of enamel.

5.2.3 Site-Specific Differences

The variations in H and E between the buccal and lingual sides of the upper 2nd molar may reflect the relative loading of the two sides during mastication. In general, both H and E are higher along the surface of the lingual cusp than along the buccal cusp. In contrast, the interior of the lingual cusp appears to have a lower H and E than the interior of the buccal cusp. The lingual cusp of molars is known to experience large loads acting normal to its occlusal surface during mastication, and this becomes more pronounced as one moves posteriorly along the tooth row [30]. Thus, H and E may be higher at this surface to shield the underlying enamel and dentine. In the intercuspal region as well, the applied loads are large at the surface. In addition, the shear stresses are also thought to be large below the intercuspal surface because a bolus of food can apply a significant bending moment to this region. Thus, it is expected that H and E are high throughout the intercuspal region to limit the degree of bending. By contrast, the buccal cusp of the upper 2nd molar typically experiences lower loads during mastication and is less likely to have a hard and stiff outer surface that shields the underlying enamel and dentine, as seen in the nanoindentation data of figure 1.

These hypotheses, linking variations in mechanical properties and tooth function, follow earlier arguments on enamel thickness. Enamel on the lingual cusps of maxillary molars is generally thicker than that on the buccal cusps [31–33]. Finite element analyses suggest that this enhanced thickness may lower tensile stresses in the intercuspal regions when the lingual cusp is loaded [34]. This suggestion may be refined further by saying that the changes in E and H as one moves from the surface of the lingual cusp to the EDJ, in conjunction with the thickness of the enamel, could result in a stress-shielding mechanism for the interior portions of the cusp. The same would hold true for the intercuspal area, where the highest measures of H and E are observed. Finite-element studies report the highest stresses due to mastication in this intercuspal region [30].

Other upper 2nd and 3rd molars (fig. 3) show similar values of H and E at the enamel surface to those of the upper 2nd molar shown in figure 1. In all cases the EDJ is noticeably weaker. However, the interior regions below the buccal and lingual cusps of the 3rd molar are harder and stiffer than the corresponding regions in the 2nd molars, particularly for the lingual cusp. Once again, these results may ultimately be tied to functional demands. Specifically, the 3rd molar is closer to the temporomandibular joint and more buccally tilted,

so it is expected to experience higher occlusal loads than an upper 1st or 2nd molar, especially on the lingual cusp [30, 35, 36]. The higher interior stiffness H and E on the 3rd molar would seem to be an effective means of coping with these higher loads.

Preliminary nanoindentation results on other teeth (premolars, incisors and canines) indicate variations in mechanical properties as large as those discussed for molars [unpubl. data]. In each case the exact distribution of mechanical properties within the enamel appears to correlate with the extent of mechanical loading experienced by the tooth during mastication. However, there appears to be an increase in the viscoelasticity (loss modulus) for the enamel of anterior teeth when compared to posterior teeth, again this may be related to their function.

5.3 Mechanics and Chemistry of White Spot Lesions

5.3.1 Origin and Importance of White Spot Lesions

White spot lesions are the earliest macroscopic evidence of enamel caries [37]. The lesions are caused by acids formed by bacterial fermentation of dietary sugars. This leads to a fall in plaque pH and dissolution of the mineral component of the tooth enamel. Under normal conditions, the demineralization process is balanced by remineralization due to diffusion of ions (Ca, P and hydroxyl) from saliva into the enamel when plaque pH returns to neutrality. However, if demineralization extent exceeds that of remineralization, then an incipient lesion is formed.

The unusual and important characteristic of a white spot lesion is that the majority of the demineralization occurs in the subsurface region. Typical lesions possess a surface layer which appears relatively unaffected by the acid attack. If lesion progression is left unchecked, then eventually the surface layer collapses to yield a cavity.

The SEM shows the surface of white spot lesions to consist of an increased number of eroded focal holes and numerous other irregular holes [38]. These areas may merge together to form larger areas of irregular cracks or microcavities. Thus, the SEM shows a distinct disintegration of the enamel surface, whereas microradiography and polarized light techniques show that the early lesion is principally a subsurface demineralization. The transmission electron microscope shows this demineralization to be diffuse, affecting both intra- and interprismatic enamel. The prism junctions appear to be sites of preferential dissolution with narrow channels occurring between prisms [39]. In these areas, crystallites are visible which are larger than those seen in sound enamel and this may represent areas of recrystallization/remineralization.

Both the macroscopic and microscopic changes in enamel associated with early carious lesions appear to be linked to specific chemical changes in the mineral, with little or no degradation of the organic matrix.

5.3.2 In vitro White Spot Lesions

White spot lesions vary from person to person, from tooth to tooth and from surface to surface, as well as with age of the lesion. Their nonstandard nature makes analysis of the lesion incredibly difficult. In order to increase the level of control over experiments with lesions, a standard method of producing in vitro lesions has been used in this study. Many of the features of white spot lesions can be mimicked by in vitro lesions, though no method completely replicates the development of the natural lesion. The lesions produced are usually referred to as 'caries-like' lesions indicating that they are not a natural white spot lesion, but an artificially produced lesion for experimental analysis. These artificial lesions provide invaluable information about the formation, the processes of demineralization and remineralization, and the composition of the lesion at different stages, in addition to being the basis for the understanding of possible treatments of the lesion.

To make the white spot lesions, human premolars were coated with nail polish except for a 5-mm square on their buccal surfaces. This ensured that demineralization always occurred at a specific location. The samples were then immersed in 200 ml methyl cellulose gel with 200 ml lactic acid solution (pH 4.6) poured on top, but separated by a sheet of filter paper. The teeth were stored in this solution at 37°C for 14 days to allow the lesions to develop. After removal from the gel, the teeth were washed and sectioned through the center of the lesions to enable the cross-section of each lesion to be viewed. The two cross-sections created for each lesion were then mounted in a low temperature cure epoxy and polished to an optical finish using 1/4-micron grit (diamond paste).

In this study the mechanical properties, hardness and reduced elastic modulus, were determined using nanoindentation, as discussed in the previous section. Nanoindentation was used to plot the mechanical properties throughout the lesion in order to evaluate the changes in hardness and reduced elastic modulus in the various regions of the in vitro caries-like lesions.

The chemical composition and structure throughout the lesions were evaluated using an SEM in conjunction with X-ray microprobe analysis (EMPA) and TOF SIMS. EMPA senses the chemistry of the sample to a depth of around 1 μm, while TOF SIMS is a surface technique that only identifies the surface atoms/ions.

5.3.3 Mechanical Properties of Lesions

The in vitro lesions consist of a mechanically weak layer at the surface (region B on fig. 7) with a hardness of $<$250 MPa and reduced elastic modulus
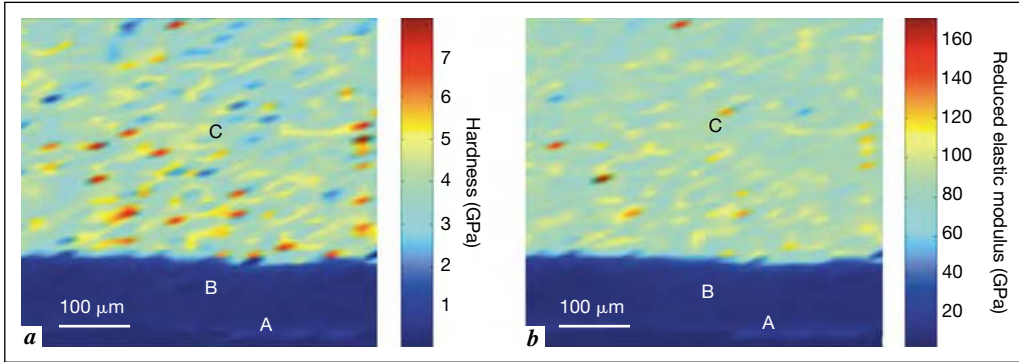
Fig. 7. Mechanical property maps of a cross-sectioned white spot lesion: (**a**) is the hardness and (**b**) the reduced elastic modulus. The regions marked A–C are described in the main text.
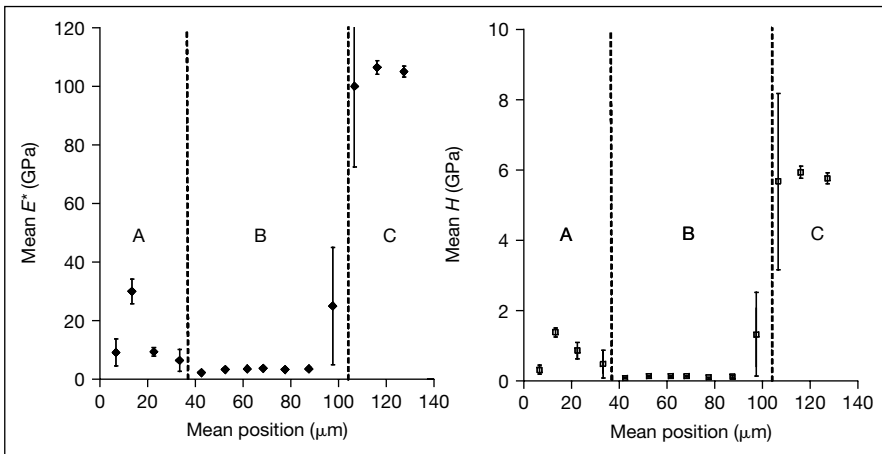
Fig. 8. Variation in (**a**) reduced elastic modulus, E^*, and (**b**) hardness, H, across the surface layers of a lesion. The labels A, B and C refer to the regions marked on figure 7.

<5 GPa. The weak layer is covered by a slightly stronger region (A on fig. 7), but this stronger surface layer is still significantly weaker than the underlying enamel (region C on fig. 7). The increased strength of the surface layer is best seen in figure 8 which shows the mechanical profile across the regions A, B and C. The increase in hardness (≈ 1.5 GPa) and elastic modulus (≈ 30 GPa) at the surface of the lesion is probably due to a slower rate of demineralization at the surface than in the lesion interior rather than remineralization.
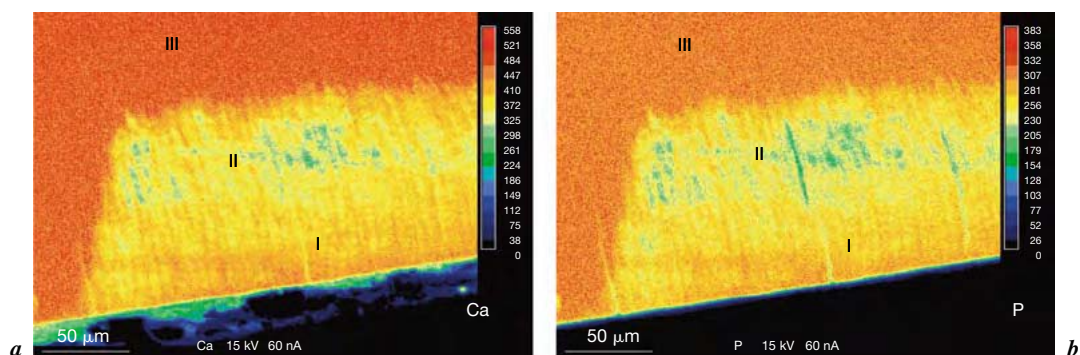
Fig. 9. EMPA image showing (**a**) calcium and (**b**) phosphate composition across a lesion. Note the high degree of demineralization in region II, but less demineralization at the surface (region I).
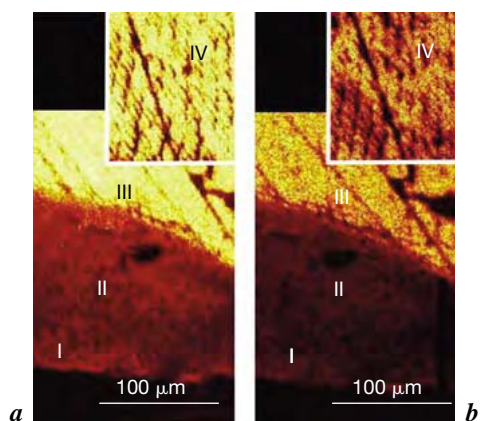
Fig.10. TOF SIMS image of a lesion showing the variations in (**a**) calcium and (**b**) phosphorus across a lesion. The insets are TOF SIMS images of sound enamel. Note that the prismatic structure of enamel is visible in the sound enamel.

5.3.4 *Chemical Analysis of Lesions*

Using EMPA (fig. 9) the chemical composition of the lesion at the surface has been determined. These data clearly show the weakened surface layer (labeled as I and II in fig. 9) to be very demineralized (low in Ca and P), but the underlying enamel (labeled as III) has a composition high in Ca and P. There is evidence of a less demineralized surface zone (region I), but the Ca and P content is still low in comparison to that of the underlying enamel.

The TOF SIMS images (fig. 10), like the EMPA data, show a small variation in the chemistry at the surface, with a subsurface layer that is generally

very demineralized (labeled II) under a slightly less demineralized layer (labeled I). Below the surface is the underlying enamel (labeled III) which has a similar Ca and P content to sound enamel (insets labeled IV). Interestingly, the underlying region of the lesion does not show the same microstructure as the sound enamel. This may be related to the process of lesion formation or may simply reflect local variations in enamel structure. The less demineralized region (region I) has a Ca and P content that is significantly less than the underlying (III) and sound enamel (IV). Care should be taken when comparing TOF SIMS data (fig. 10) with EMPA (fig. 9) or microradiography data. TOF SIMS is a truly surface technique; it works by removing the surface layer of atoms/ions and then measuring the mass and quantity of the ions. Thus, it counts the number and species of the atoms per unit area of the surface. EMPA and microradiography methods sense composition over a volume. The variations in porosity within a lesion may affect the volume-dependent methods and give different absolute values for Ca/P content when compared to surface techniques.

5.3.5 Implications of Mechanical and Chemical Measurements

There is a correlation between the mechanical and chemical data of figures 7–10. It is clear from the multilayer or lamellar structure of these in vitro lesions that they are formed by a complex demineralization process that cannot be explained by simple, diffusion-based models. The surface layer, which is extremely weak, has lost almost all of its Ca and P, except for a very small amount close to the surface. This region close to the surface is stronger than the body of the lesion, but still very weak when compared to the underlying enamel. The body of the lesion is extremely compliant and mechanically very weak. The weak interior and surface layers of the lesion make it particularly prone to damage when the surface is mechanically loaded. Collectively, the mechanical, chemical and structural data indicate that even the less demineralized surface zone (A on fig. 7, 8) does not have the same microstructure or mechanical strength as sound enamel.

The present in vitro lesions were formed under controlled demineralizing solution conditions. Lesions may also be produced under so-called pH-cycling conditions, which reproduce the alternating periods of demineralization and remineralization that occur in the mouth (see chapter 3). An interesting topic for future research would be to investigate the changes in mechanical properties when partially demineralized enamel is subjected to net remineralizing conditions during which the surface zone will become more pronounced. Such work should be able to show how far it is possible to restore the mechanical properties of the surface enamel to its former strength.

5.4 Nanomechanics and Morphology of Salivary Pellicle

5.4.1 Composition of Pellicle

Saliva is the body's natural protective mechanism against decay. It contains salivary proteins which adsorb strongly onto the teeth to form a layer which is referred to as the salivary pellicle.

The chemical composition of old pellicle samples has been reported to be 46% amino acids, 2.7% hexosamines and 14% total carbohydrate [40]. Amino acid compositions of pellicle collected from different teeth are virtually identical [41] and compositions of 2-hour acquired enamel pellicle have been found to be consistent over a period of 24 months [42]. In contrast, 24-hour pellicles exhibit significant variability when subjects have consumed a normal diet. An amino acid composition similar to that of enamel pellicle has been found for the film on the sides of maxillary dentures [43]. These results suggest that most oral surfaces bathed with saliva acquire a protein coating with a composition similar to that of enamel pellicle [44].

The major salivary proteins in pellicle are secretory IgA, acidic proline-rich protein (PRP), cystatin SA-I, high-molecular-weight mucin MG1, lactoferrin, lysozyme and amylase.

5.4.2 Growth of Pellicle

The growth of pellicle seems to occur in two distinct stages. In the first stage, an initial organic covering forms on enamel within minutes of exposure to the oral environment. In the second stage the thickness of this layer increases to a maximum after approximately 2 h [45–47]. Tests on pellicle that is several days old have shown it to remain at about the same thickness as 2-hour pellicle [48].

The two-stage growth process of the pellicle is of importance to the structure and function of the pellicle. The first step has been explained by an initial adsorption of discrete proteins to the enamel surface, whereas the second step is the adsorption of salivary protein aggregates in the form of micelle-like structures that move more slowly towards the interfaces, and hence give a stepwise increase in the pellicle thickness [47]. The latter structures are believed to account for the globular surface morphology of acquired salivary pellicle.

5.4.3 Pellicle as a Chemical Barrier

One of the main functions of salivary pellicle is protection of the enamel from acidic attack. For example, recent studies have demonstrated that the presence of a salivary pellicle drastically reduced erosion of enamel by an acidic cola drink [49] and by orange juice [50, 51]. The pellicle appears to act as a

semi-permeable barrier between plaque fluid and the enamel surface. Reduced diffusion fluxes [52] lead to slower rates of mineral dissolution.

The pellicle seems to need to go through a maturation process in order for it to form a protective barrier against acidic solutions [41, 53, 54]. Enzymes are readily available within the oral cavity with the potential to stabilize the maturing pellicle through cross-linking.

5.4.4 Pellicle as a Lubricant

As well as chemical protection, salivary pellicle is believed to be important in reducing wear by acting as a lubricant. Lubrication is very important during the act of mastication, due to the large forces applied and the brittle nature of enamel. Although the pellicle layer is typically $<1\,\mu$m thick, it acts as an absorbent cushion which helps prevent the teeth from cracking and chipping. This is also important for parafunctional activity, such as grinding and clenching of the teeth, where the pellicle serves to minimize the abrasion caused.

The viscoelastic properties of pellicle are vital to its function as a lubricant. Certain chemicals, such as tannins, which are common in foods and drinks, may alter the structure, viscosity and appearance of pellicle. This can lead to a change in its performance as a lubricant. In addition to this, the removal of the surface layer of the pellicle by the abrasive process of toothbrushing may not be as effective due to the changed nature of the pellicle. This can mean that the tannin-stained pellicle on the tooth surface is not removed during normal day-to-day brushing. Our study, described below, has focused on using nanoindentation and scanning probe microscopy techniques to examine the effects of tannins on pellicle viscosity and morphology.

5.4.5 Pellicle and Tannins

Many foods obtained from plants contain tannins. These are phenolic compounds that bind proteins, such as mammalian enzymes. It has been shown in vitro that tannic acid significantly reduces the lubricating qualities of human saliva both by decreasing its viscosity and increasing friction [55]. This effect depends on the presence of salivary PRPs, which have a high affinity for tannins [56]. It has been shown that the introduction of tannins into the diet can stimulate the production of PRPs in the saliva of rats [57]. PRPs are a major component of salivary pellicle, and thus it would not be surprising to find that dietary tannins affect the viscosity of pellicle.

For this study, pellicle samples were formed in vivo on polished samples of human enamel and soda-lime-silica glass (microscope slides). The samples were placed in a specially designed oral device that clipped onto the upper molars in the human volunteer's mouth. To keep the pellicle growth consistent, each volunteer thoroughly cleaned their mouth using a commercial toothbrush
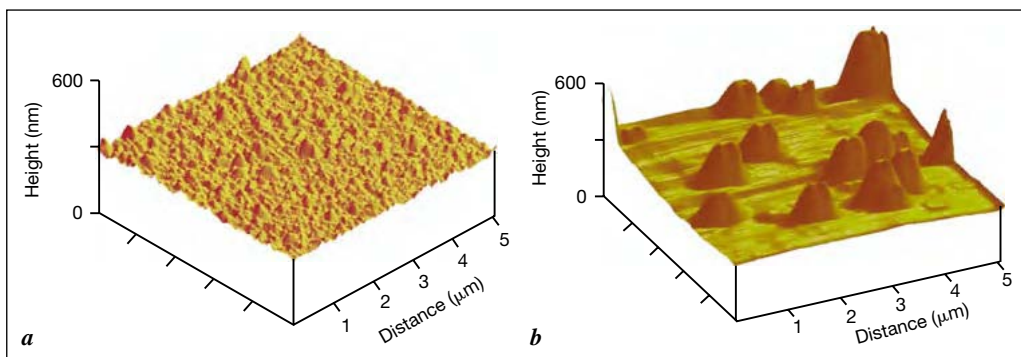
Fig. 11. (*a*) AFM image of 2-hour salivary pellicle formed in vivo on polished enamel. (*b*) AFM image of the same salivary pellicle, but not the same location, as (*a*) after controlled exposure to a tannin-containing solution (black tea).

and toothpaste, then allowed the mouth to equilibrate for 2 h. An intraoral device was then placed in the mouth for the desired amount of time to allow pellicle to grow (typically 2 h), before being removed and rinsed in deionized water. At no point during the experiment was intake of food or beverages allowed.

The structure of all the 2-hour pellicles was found to be consistent (fig. 11a), and hence this time period was used as the standard for all the pellicle-tannin interaction tests. For pellicle on both human enamel and glass substrates, the globular structure of the pellicle was found to be the same as that reported in the literature.

Tannins react with PRPs leaving a red/brown stain on the pellicle, in addition to changing its mechanical properties. AFM images of the structure of tannin-reacted pellicle show that the morphology of the pellicle can be dramatically changed with the interaction of tannins from a tea solution (fig. 11b). This change in physical structure implies that cross-linking of the proteins is occurring, causing the configuration of the globules to take a lower energy structure. The increase in globule size is probably due to elastic strains created by cross-linking within the proteins.

Pellicle and tea-immersed pellicle were analyzed using nanoDMA (dynamic mechanical analysis) to see if the tannins had an effect on the viscoelasticity of the pellicle. NanoDMA is a technique used to study and characterize mechanical properties in viscoelastic materials. The method is an extension of nanoindentation testing [58, 59]. An analysis of the nanoindentation load-depth curve gives the hardness (*H*) and reduced elastic modulus (*E**), provided the area of contact, *A*, between the indenter tip and the sample is known [13]. By
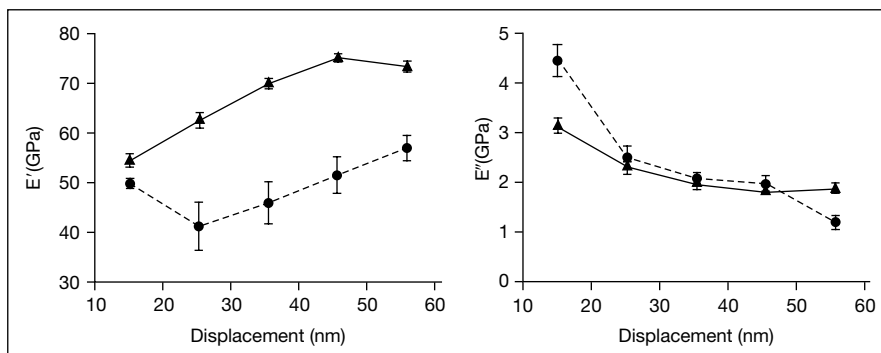
Fig. 12. NanoDMA data for 2-hour in vivo salivary pellicle before (continuous line and triangles) and after (dashed line and circles) controlled exposure to a tannin-containing solution (black tea). E' is the storage modulus and E'' is the loss modulus.

applying a small oscillatory force during an indentation, time-dependent properties can also be found. The phase difference and amplitude of the resulting displacement oscillation relative to the load modulation can be used to obtain the viscoelastic properties (storage and loss modulus) by using a damped oscillator model. This is the essence of the nanoDMA methodology [60, 61].

NanoDMA has been successfully used in characterizing viscoelastic properties for a range of polymeric materials, including polyethylene [62], bis-maleimide and thermoplastics [63]. There have also been recent reports of nanoDMA being used to examine biological materials such as hairs and whiskers [64]. However, the data presented in this section are amongst the first ever obtained for a biofilm.

The storage modulus (E') is the measurement of energy stored during deformation and is a measure of the solid-like, or elastic, portion of the pellicle's deformation. The loss modulus (E'') is the measurement of energy lost during deformation due to viscous drag. From the nanoDMA results (fig. 12) we can see that the interaction of tannins increases the ratio of the loss modulus to the storage modulus. This implies that the pellicle is less viscous after the tannin interaction and more prone to time-dependent deformation. In fact, it resembles a stiff elastomer ('rubber-like') in terms of its mechanical properties. These properties are crucial in determining the efficiency of toothpaste abrasives for removing stained pellicles.

5.4.6 Effect of Tannins on Pellicle Mechanics and Morphology

The morphology and mechanics of salivary pellicle show substantial changes when exposed to tannin-containing drinks such as tea. Increases in the

ratio of loss modulus to storage modulus enable the pellicle layer to stretch and then recover rather than being abraded by the action of dentrifices. The increased size of the pellicle globules may also affect the efficiency of abrasive dentrifices. However, understanding how the surface morphology affects tribological processes is complex. It is possible that the elastomer-like behavior of tannin-stained pellicle creates a contact geometry between contacting teeth, or between an abrasive particle and a tooth, that resembles the model for adhesive contacts developed by Fuller and Tabor [65]. This model is based on the so-called JKR model [66] for adhesive contacts, but incorporates surface roughness as a series of parabolic surface asperities. This model may be particularly appropriate for the elastomer-like stained pellicle, as the JKR model for adhesion was originally verified on rubbery materials. This model is not as appropriate for the unstained pellicle as it is less like an elastomer, though it does still have a globular surface morphology. Overall, the changes in morphology and mechanical behavior provide a clear insight into why the tribological properties of stained pellicle are significantly different from unstained pellicle, and why it is harder to remove from the enamel surface with a dentifrice.

5.5 Summary

During early studies, dental enamel was assumed to be a homogeneous material with uniform mechanical properties. Now, it is generally recognized that the mechanical response of enamel depends upon location, chemical composition and prism orientation. The results presented here show that over the axial cross-section of maxillary 2nd molars local variations in mechanical characteristics correlate with changes in enamel chemistry, microstructure and prism alignment. The mechanical properties have been characterized with nanoindentation techniques, the chemistry has been identified with EMPA and the microstructure with SEM. A substantial change has been found in mechanical properties traversing from the enamel surface, $H > 6\,GPa$ and $E > 115\,GPa$, to the EDJ, $H < 3\,GPa$ and $E < 70\,GPa$. These properties appear to show the strongest correlation with changes in the average chemistry of enamel. The mechanical properties of the enamel also differ from the lingual to the buccal side of the molar. At the occlusal surface the enamel was harder and stiffer on the lingual side than on the buccal side. The interior enamel, however, was softer and more compliant on the lingual than on the buccal side. These variations also correlated with differences in average chemistry and might be related to differences in function of the lingual and buccal sides of maxillary 2nd molars.

The mechanical properties of the enamel surface have been found to be highly dependent on the chemistry of the oral environment. Frequently, large variations in mechanical behavior are seen due to changes in the surface chemistry. Nanoindentation and AFM have been used to examine the effects of surface chemistry on the mechanics of the enamel surface. These methods provide mechanical characterization with unprecedented spatial resolution. It has been found that the demineralizing effect of acid leads to the development of a white spot lesion for which there is a relatively strong layer near the enamel surface but a very soft interior. These variations in mechanical behavior have been correlated with EMPA and TOF SIMS data on the Ca and P composition. In the future, use of nanoindentation, to examine lesions which have been treated with remineralizing chemicals, will provide a valuable quantitative measure of their potential to restore the enamel's mechanical function. Also at the enamel surface, a layer of salivary pellicle is acquired by adsorption of salivary proteins and other organic matter in the oral cavity. The pellicle shows large variations in its mechanical properties and growth, which appear to be related mostly to changes in the chemistry of the oral environment, for instance due to diet. Notably, beverages associated with extrinsic staining of enamel (e.g. tea) have been shown to dramatically change the morphology and viscoelasticity of salivary pellicle, hence making the enamel surface much harder to clean with a dentifrice. There is apparently little effect on the properties of the pellicle due to changes in the substrate on which it forms. The pellicle that forms on replacement materials, such as glass, does not differ noticeably from that grown on enamel.

Acknowledgments

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Plaque as a Reservoir for Active Ingredients

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6.1 Introduction

The roles of plaque and saliva in the initiation and progression of dental caries are summarised schematically in figure 1. A central feature is the generation of organic acids, such as lactic acid, by acidogenic plaque bacteria following the dietary intake of carbohydrates such as sucrose. As discussed in Chapter 1, saliva performs two direct functions in order to combat enamel dissolution by these acids: (a) the continuous flow of saliva acts to clear the acids from the mouth and (b) the supply of a number of diverse salivary constituents that have 'anticaries activity'. The latter constituents can act on the acids themselves, via buffering or neutralisation, on the bacteria, via inhibition of the metabolic processes involved in acid production, and on the enamel, by maintaining chemical supersaturation in the adjacent plaque fluid. A key indirect function of saliva is as a medium for the transfer of potentially active therapeutic agents, such as fluoride (F), to the site of action.

The physical presence of plaque mediates all the above salivary functions through its influence on ionic and molecular transport, whilst plaque components also provide binding sites for many salivary constituents and therapeutic agents. This plaque 'reservoir' function is the theme of this chapter.

First, we describe the chemical composition of plaque fluid in relation to caries, then the role of plaque structure. Next, we discuss the influence of F retained in plaque, including site-to-site differences, followed by the effect of treatments that seek to deposit plaque calcium (Ca) and/or inorganic phosphate (Pi). A combination of small sample volumes and low constituent concentrations typically leads to high measurement variability that results, in turn, with authors often having difficulty in discriminating between subject groups.
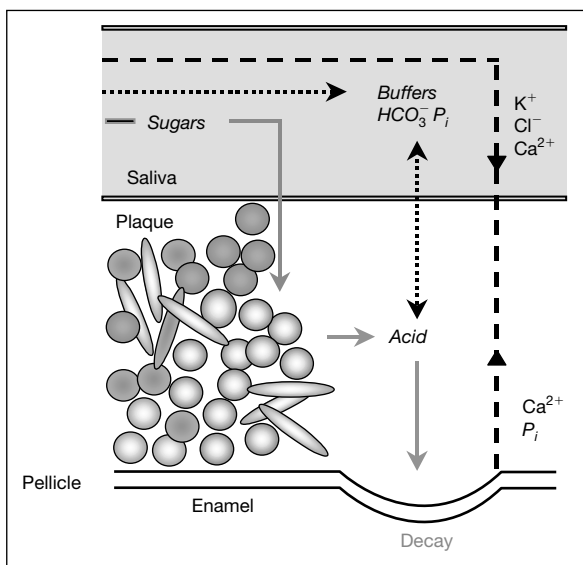
Fig. 1. Schematic diagram of the plaque/tooth interface.

6.2 Chemical Saturation of Enamel Minerals in Dental Plaque in Relation to Caries Susceptibility

De- and remineralisation are two dynamic processes of dental caries, in which chemical composition plays a key role. The driving force for de- and remineralisation of tooth mineral is the degree of saturation (DS) with respect to dental minerals in adjacent liquid, i.e. plaque fluid in the caries situation.

Plaque fluids can be isolated by high-speed centrifugation and analysed by, for example, capillary electrophoresis and ion chromatography. Certain species, such as H^+ and F^-, are usually determined by ion-selective electrodes. The main inorganic anions are chloride and Pi, whilst short-chain organic acids include lactic, acetic, propionic, succinic, formic, pyruvic and butyric acids. The main cationic components are ammonium, potassium, magnesium and Ca. Although it cannot be concluded that any individual component plays a key role in the caries process, studies have shown that some components are more related to caries susceptibility than others.

Moreno and Margolis [1] developed a computer program, which could be used to calculate the DS with respect to many CaPi phases. To calculate the DS in plaque fluid, the main inorganic ions, especially the concentrations of Ca and Pi, the ionic strength and the pH have to be measured. Clinical studies

showed that the DS with respect to enamel could be a more sensitive parameter to distinguish caries activity than concentrations of individual constituents.

In his 1990 review of plaque fluid composition, Margolis [2] described studies from his own and other groups that sought to differentiate between caries suspectible (CS) and caries-free (CF) individuals. He emphasised his finding that ammonia and other pH-rise factors appeared prominent in distinguishing between plaques collected from such groups. He also noted that in resting plaque fluid more changes occurred in the inorganic content on average than in organic acid composition, which appeared to remain relatively constant.

At the same meeting, Tatevossian [3] provided a useful critical review of the value and potential pitfalls of studies of plaque fluid undertaken up to that time. He noted that, because of the small amounts of plaque that are typically collected in such studies, a majority of researchers analyse pooled samples. He regarded solubility calculations based on Ca, Pi and other ion concentrations obtained in this manner not to be meaningful. In contrast, whilst acknowledging site-specific sampling to be the ideal, Margolis [2] claimed that his findings noted above, and the degree of consistency observed between results from different laboratories, demonstrated that useful information could indeed be obtained from samples of pooled plaque.

In a further study of pooled plaque samples from groups of caries-positive (CP) and CF individuals, Margolis and Moreno [4] observed differences between the groups for pH, lactate and total Ca (in whole plaque but not free Ca ion concentration in the fluid), both before and after a 10% sucrose rinse. After the sucrose treatment, both lactate and Ca concentrations increased as pH decreased. Mean degree of supersaturation with respect to enamel, DS (en), estimated from analyses of the overall ionic composition of the plaque fluid, tended to be higher in the CF subjects. Margolis et al. [5] reported similar findings for plaque fluid from exposed root surfaces. The authors suggested that the availability of mineral ions, such as Ca, within plaque may play an important role in controlling enamel mineralisation. Tanaka and Margolis [6] later provided in vitro evidence consistent with this hypothesis.

In a recent study by Gao et al. [7] using similar methodology, subjects were divided into three groups: CF, caries active (CA, with active carious teeth) and CP (with filled teeth because of past caries experience). The subjects were asked to fast overnight and stop oral hygiene for at least 48 h. Pooled plaque samples were collected after a 1-min sucrose rinse and analysed for inorganic ions and organic acids. The DS values for each individual plaque sample were estimated with the computer program of Moreno and Margolis [1]. Of all the parameters, only the DS values could distinguish the three groups clearly (table 1).

Table 1. Composition [mean (SD)] in plaque in relation to caries activity [7]

Composition	Group	Baseline	3 min after sucrose rinse	7 min after sucrose rinse
DS	CF	3.07 (1.54)	0.74 (0.37)	1.47 (0.53)
	CP	2.72 (0.87)	0.95 (0.35)	1.01 (0.52)
	CA	2.89 (1.11)	0.49 (0.16)	0.47 (0.36)*
pH	CF	6.58 (0.41)	5.59 (0.20)	5.96 (0.18)
	CP	6.68 (0.46)	5.68 (0.15)	5.89 (0.17)
	CA	6.66 (0.33)	5.37 (0.11)	5.30 (0.28)*
Ca	CF	1.43 (1.12)	4.75 (1.21)	4.04 (0.58)
	CP	2.95 (1.81)	5.70 (3.35)	3.57 (1.16)
	CA	3.16 (2.83)	5.47 (1.01)	3.64 (1.26)
PO_4	CF	12.70 (7.30)	9.40 (2.20)	8.90 (1.20)
	CP	9.70 (4.00)	9.70 (2.60)	7.90 (1.20)
	CA	11.00 (2.00)	9.40 (0.80)	8.50 (1.70)
Lactate	CF	5.80 (5.00)	37.30 (3.30)	30.20 (4.80)
	CP	6.90 (4.50)	28.30 (7.70)	22.50 (11.50)
	CA	5.60 (4.10)	33.10 (5.10)	35.30 (17.60)

CF = caries free, n = 6; CP = with past caries experience, n = 7; CA = with active caries, n = 9.

*$p < 0.05$, compared with CF, by Tukey's test for comparison made at a given time point.

The results demonstrated that within plaque at rest, i.e. without any sugar exposure in 2 h after meals, there was no significant difference for any individual fluid constituents among the groups of subjects. However, after sugar exposures, for example, 7 min after a 1-min sucrose rinse, pH and lactic acid in the plaque fluids of the CA group were significantly different compared with plaque fluid from CF people. In particular, the mean pH value in plaque fluid of the CA group was below the so-called critical pH for a longer time than in fluid of the CF group and of the CP group. Dong et al. [8] reported a similar trend. This may suggest high acid production in CA subjects or, more likely, suggest longer acid retention.

With regard to DS, DS = 1 implies a saturation condition in plaque fluid, DS < 1 undersaturation and DS > 1 supersaturation. In all subject groups the plaque fluid was saturated with respect to tooth enamel before sucrose exposure. Immediately after sucrose exposure, the plaque fluid was undersaturated for all subjects, whether they had caries or not. However, for those who were CA, the undersaturated state persisted for longer after sucrose exposure, because of retention of the acids produced. This would at least partially explain the high risk of demineralisation in CA subjects as shown in table 1.

6.2.1 Effect of Oral Hygiene on Plaque Composition

Dental plaque can accumulate when oral hygiene is poor. Long-term accumulated plaque may be more condensed and may express different bacterial metabolic processes. However, little is known about the composition of dental plaque from people who do not practice any oral hygiene. Studies of plaque composition in people without oral hygiene should help us to understand the effects of oral hygiene intervention on plaque metabolism. Recently, Gao and coworkers [9] obtained plaque samples from a group of Chinese peasants who had never brushed their teeth in their life, neither using F nor any other oral hygiene products. The subjects were divided into CF and CP subgroups. Plaque samples were collected before and after a sucrose rinse. Inorganic ions and organic acids were analysed. The main finding from this study was that formic acid concentrations in this group of people without oral hygiene were higher (CF group: 4.82 ± 4.85 mmol/l at baseline, 11.15 ± 6.72 mmol/l at 7 min after sucrose rinse; CP group: 2.97 ± 3.13 mmol/l at baseline, 15.92 ± 12.09 mmol/l at 7 min after sucrose rinse) than found in their previous studies (of subjects with conventional tooth-cleaning habits [10]) and in some of the published work by other investigators [1], which demonstrated a very low level of formic acid (mostly below the detection limit). Moreover, the concentration of formic acid increased significantly after sucrose exposure. Whether this was caused by a different microecology is unknown and needs further investigation.

6.2.2 Change of Plaque Composition in Relation to Caries Experience in a Group of Patients after Radiation Therapy in the Neck and Head Regions

Patients with cancer in the head and neck area often receive high dosage radiation therapy, which damages saliva gland function and can reduce salivary flow rate dramatically. Caries often occurs in these patients in a very short time after the treatment. Whether this is simply because of reduced salivary flow or for other reasons remains unclear. Thus, Wang et al. [11] designed studies to analyse plaque composition in such patients before and after receiving radiation therapy. The results were compared with normal subjects. Three groups of subjects were observed:

- Rampant caries group (RC group): DMFT $\geq$ 15, who had significantly reduced salivary flow rate and had received radiation therapy or were patients diagnosed with Sjogren's syndrome. All patients complained of a dry mouth.
- CP group: generally healthy with normal salivary flow rate, DMFT $\geq$ 10, with active caries.
- CF group: generally healthy with no caries.

Table 2. Lactic acid (mmol/l) in plaque fluid [mean (SD)] after 1-min sucrose rinse (data taken from [11])

Subject group (n)	Before sucrose	7 min after	15 min after	30 min after
CF (18)	6.60 (4.45)	22.50 (11.37)	10.79 (5.28)	4.08 (2.36)
CP (15)	7.69 (5.47)	25.47 (14.98)	15.62 (11.84)	9.73 (6.91)*
RC (22)	7.15 (5.07)	27.16 (10.20)*	17.93 (10.58)	14.61 (5.60)*

CF = caries free; CP = with past caries experience; RC = rampant caries.
*$p < 0.05$, by Tukey's test for comparison with CF group.

Plaque samples were collected before and 7, 15, 30 min after a sucrose rinse for each individual. Lactic, acetic, butyric, formic, propionic, pyruvic and succinic acids were determined by capillary electrophoresis. By analysis of the results, only lactic acid showed a significant difference in the RC group compared with the other two groups (table 2). Of note, the elevated lactic acid concentration was sustained for longer in the RC group than in the other groups, possibly caused by the reduced salivary flow, and may be responsible for the serious caries.

6.2.3 The Influence of Plaque Structure on Acid Production and Clearance

In the context of a chapter on plaque as a reservoir for active ingredients, plaque structure has an important influence on the penetration and clearance of such materials and also of various other species involved in the caries process. We have discussed in previous sections the thermodynamic approach to caries susceptibility adopted by Margolis et al. [1, 4–5] that focuses on calculations of the DS of the plaque fluid with respect to dental enamel based on extensive chemical analyses of plaque samples. Dawes, Dibdin and their co-workers [12–19], on the other hand, have modelled essentially the kinetics of the saliva-plaque system to compute Stephan curves within plaque and at the enamel surface following sucrose exposure. Sucrose (and related highly water-soluble species such as glucose) strictly speaking are not retained in plaque, but are either rapidly cleared from the mouth by saliva or converted to other molecules by plaque bacteria. The H^+ ions that determine pH are one product of such conversion processes and are retained to an extent.

The latter mathematical modelling work has done much to increase our understanding of the role of plaque in the dynamics of the caries process. Dibdin has provided a recent overview of this topic [12], which focuses more

on the modelling process than on applications. His original model [13, 14] made no assumptions as to the velocity or direction of flow of saliva over the plaque surface and the predominant effect was a generalised mixing of the local salivary film with the main pool of saliva in the mouth as a whole. Dawes and coworkers [15, 16], in contrast, assumed that there was directional flow across the plaque surface and saliva composition consequently changed with distance as a function of the flow rate at constant film thickness. Subsequent collaborative work showed that both models yielded similar results when applied to the effects of chewing gum on plaque pH [17]. Of importance, the last-mentioned work highlights potential site-specific differences in Stephan curves after sucrose exposure, consistent with expectation, which are relatively independent of the chosen model.

Recent models take into account not only the nature of the saliva film and its composition but also the diffusion and reaction of salivary components within plaque, metabolic processes of plaque bacteria, salivary and plaque buffering, Ca^{2+} ion binding, etc. Earlier work [18] had emphasised the role of plaque thickness on the shape of the Stephan curve: for a given sucrose pulse, minimum pH values at the enamel/plaque interface were predicted to be lowest for plaques of thickness intermediate between thin (0.1 µm) and thick (2 µm) plaques. The more recent calculations, whilst generating deeper and more prolonged pH falls, have shown that these differences remain important [19].

Certain of the studies reported in section 6.2 suggested that the plaque pH profiles of CS subjects displayed more prolonged pH falls on average than those of CF subjects. These falls are consistent with the above theoretical findings, in so far as an important risk factor for CS subjects is poor oral hygiene (Chapter 1).

6.3 Fluoride

The various modes of action of F as an anticaries agent have been discussed in detail elsewhere [20, 21]. The delivery and retention of F at, or close to, the site of action is regarded as an important feature of the successful application of the agent [22, 23]. In particular, the maintenance of an elevated, even if low, F concentration adjacent to the tooth surface has long been believed to be the key to achieve optimal caries control [24].

6.3.1 Site-Specific Differences in Oral Fluoride Concentration
Weatherell et al. [25] were probably the first workers to demonstrate the inhomogeneous distribution of F around the mouth following a F treatment, even when that treatment had been applied as a mouthrinse (1,000 ppm F

sodium fluoride; NaF). Essentially, they found higher F concentrations in the upper vestibule than in the lower vestibule. Sites where F clearance was relatively rapid were associated with regions close to salivary duct orifices and/or where salivary flow was expected to be significant, whereas F retention was most pronounced at sites which could be envisaged as regions of salivary stagnation. In this study, samples of fluid were collected from various oral sites using small paper points. Hence, the source of the F subsequently analysed was the salivary film adjacent to the various oral surfaces. Nevertheless, such data are useful to compare with later, more direct measurements of F in plaque.

A study using a saliva collection procedure that also utilised absorbent paper pieces was reported by Zero et al. [26]. They were also able to show variations in F levels around the mouth, although specific site-to-site comparisons were qualitatively different from those reported by Weatherell et al. [25] presumably because of differences in other aspects of the methodology.

F in site-specific plaque samples was investigated by Vogel et al. [27]. These researchers monitored F in individual samples of plaque fluid using a specially developed micro-analytical technique [28]. Their results were largely consistent with those of Weatherell et al. [25]. F concentrations following application of a NaF rinse (910 ppm F) were highest in plaque taken from the upper incisors and were similar in plaque taken from molar sites on either side of the mouth. Vogel et al. [27] noted that the upper incisors had been observed to be a site with slow salivary clearance in a study by Lacomte and Dawes [29].

Vogel et al. [27] found that plaque fluid F concentrations were significantly higher than corresponding whole saliva F values, with which they were highly correlated, over a 60-min period following mouth rinse application. This difference was also found at baseline. The shapes of the plaque fluid F and saliva F versus time curves suggest that F clearance is slower from plaque than from saliva. These differences may reflect more specific F binding at plaque reservoir sites [30] or simply the more restricted diffusion of F out of the plaque matrix (see section 6.2.3).

The same group also reported finding a high level of F in plaque fluid relative to saliva in a second study [31]. However, in this work individual plaque fluid F concentrations and corresponding saliva values were not correlated. This is no doubt because in the second study the applied F rinse was quite different from that used in the first study, comprising two separate solution components that were mixed on application and designed to precipitate calcium fluoride (CaF_2).

The site-specificity of plaque fluid F reported by Vogel et al. [27] is re-enforced by the study of Ekstrand [32], who used the same careful methodology. Ekstrand's data clearly show the ranking of F concentration: upper incisors > lower molars > upper molars > lower incisors. F clearance from

plaque was again slower than from saliva, a finding also reported by Zero et al. [33].

Site-specific sampling was also employed by Bottenberg et al. [34] in their study of oral F retention and clearance after application of a bioadhesive F-releasing tablet. In this study, subjects each placed a tablet at one of two locations in the mouth and then collected saliva film samples from four different sites for up to 4 h using miniature absorbent sponges. F concentrations were lower on average with a tablet placed in the centre of the palate compared with in the lower vestibule. The authors attributed this to either greater abrasive removal of tablet material by the tongue, preferential direct swallowing of released F because of the closer proximity of the pharynx, or both. F concentrations at anterior sites were higher than those adjacent to upper or lower molars, consistent with the salivary clearance studies of Lacomte and Dawes [29].

6.3.2 Influence of Different Treatments on Plaque Fluoride

Ekstrand's work [32] also highlighted the greater F levels in plaque found after application of an aqueous NaF rinse compared with corresponding values following a sodium monofluorophosphate (Na_2FPO_3, NaMFP) rinse of equivalent F content. This difference agrees with the results of a previous study of plaque F after use of F dentifrices reported by Duckworth et al. [35]. However, the latter authors attributed the effect to the presence of the surfactant sodium dodecylsulphate in the dentifrice formulation, which is known to inhibit the enzymatic breakdown of FPO_3^{2-} ions to F^-. The greater difference observed in the Ekstrand study is, in part, likely to be because he only analysed for F^- ions rather than total ionic F. A further potential cause of a difference between the two species is the slower diffusion of FPO_3^{2-} ions in plaque than of F^- ions, as exemplified by the work of Pearce and Dibdin [36].

A study by Vogel et al. [31] is of interest because it showed that a rinse designed to precipitate CaF_2 yielded significantly more F in both plaque and plaque fluid, even 2 h after application, than a more conventional rinse that contained F^-. In a later study the same workers showed that these qualitative differences persisted overnight [37].

Kato et al. [38] also demonstrated the ability of a precipitating mouth rinse to elevate F levels in plaque. They utilised a so-called 'mineral-enriching' solution developed by Pearce and Nelson [39] that contained Ca, Pi and urea as well as F (in the form of NaMFP). By using an in situ plaque-sampling device [40], concentrations of F could be determined ex vivo at different plaque depths. Significant increases in F were observed in the outer and middle plaque layers following repeated daily use of the mouth rinse compared with values in plaque exposed to a control rinse. Qualitatively similar increases were found for plaque Ca and Pi.

Earlier work of the same group suggested that F from a NaF rinse was able to penetrate to the inner plaque layer easily [41]. Kato et al. [38] propose that the different F profile found after the mineral-enriching rinse may indicate that the F is associated with a CaPi-containing deposit. These authors were also able to show that plaque F correlated positively with salivary Ca [38], which (in the case of the control rinse or a rinse that contained free F ions) would be consistent with a mechanism of F binding to plaque bacterial cells via Ca^{2+} proposed by Rose et al. [30].

Duckworth et al. [42] were able to demonstrate that plaque F increased in a dose-dependent manner in a study involving a series of mouthrinses of increasing NaF concentration (0, 100, 250, 1,000 ppm F) that were each used regularly for about a month. Moreover mean plaque F was significantly correlated with corresponding values of mean saliva F concentration. These measurements were made on plaque samples collected at least 18 h after last treatment use, and therefore represented F retained in one or more oral F reservoirs.

6.3.3 Effect of Fluoride on Plaque Acid Production

The most important modes of action of F to control caries are believed to be the inhibition of demineralisation and the promotion of remineralisation of enamel [20, 21]. Effects of F on plaque bacteria have long been known but most supporting evidence has been obtained in vitro.

The effect of F on plaque acid production in vivo has been demonstrated in several recent studies. In the work of Gillman et al. [43], for example, 11 subjects rinsed for 1 min with 10 ml of either 0.2% NaF solution or water and then either 2 or 60 min later rinsed with 10 ml of 10% sucrose solution for 1 min. The treatments were carried out in a randomised crossover design. Smooth surface plaque samples were collected before the treatment rinse and 15 min after the sucrose rinse, after which they were analysed for plaque acids by capillary electrophoresis (for details, see [44]). Acetate was the predominant acid in the pre-rinse samples, whereas lactate was the major acid induced by the sucrose rinse, as observed by other authors [4, 44–47]. Figure 2 shows that at both time points, lactate production in plaque was significantly inhibited after the F rinse compared with after the water control rinse by about 50%, consistent with the findings of Oliveby et al. [46]. Vogel et al. [47] also reported a decrease in plaque lactate following a sucrose rinse given 1 h after an experimental high-F deposition rinse compared to a 'no treatment' control.

In a separate study, lactate concentration 15 min after a sucrose rinse was found to be 20% lower on average in interproximal plaque than in smooth surface plaque collected at the same time from the same subjects [Gillman and Green, unpubl. data]. This finding is consistent with the view that the interproximal plaque was less accessible to the sucrose.
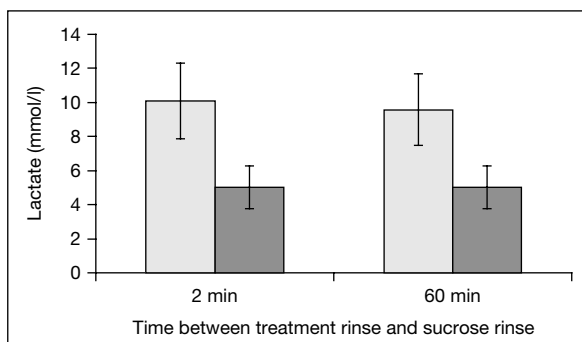
Fig. 2. Effect of pre-sucrose rinses on lactate in plaque collected 15 min after a 10% sucrose rinse (mean ± SE; n = 11; 10-ml rinses). Light bars = water control; dark bars = 0.2% (48 mmol/l) NaF solution.

6.3.4 Oral Soft Tissues as a Reservoir for Fluoride

Studies involving fluoridated dentifrices [48] suggested that, whilst salivary F clearance within 3 h after a single application could be explained on the basis of a single oral F reservoir, more than one oral F reservoir had to be invoked to explain F retention over longer periods. Duckworth and coworkers [22, 49] have maintained that the oral soft tissues are the most likely site for the primary F reservoir because of the relatively large exposed area of such tissue and the ability of F to associate with, and be released from, such tissue.

Zero et al. [26] highlighted the role of the oral soft tissues in providing a reservoir for retained F by showing that F levels in samples taken from a panel of edentulous subjects were similar to, or indeed higher than, those in samples taken from a panel who were fully dentate. Moreover, F levels monitored adjacent to specific soft tissue sites correlated well with corresponding whole saliva F concentrations.

Despite the likely major source of salivary F being soft tissue, it is undoubtedly true that the F in plaque plays a key role in the anticaries action of F. Vogel et al. [27] suggested that the oral mucosa could not act as a source of F for plaque because of their finding of a decreasing F concentration gradient from the plaque fluid to the intervening saliva. However, this conclusion cannot be deduced with certainty from their data because their saliva samples were not taken from sites adjacent to the plaque and, as demonstrated by Weatherell et al. [25], F concentrations *in the salivary film* vary around the mouth. Indeed, the lead author of [27] now believes in such a possibility [Vogel, pers. commun.].

6.4 Calcium and Phosphate

In section 6.3, we described studies where the primary aim was to boost the F level in plaque as an anticaries measure. Complementary information is provided by studies of 'plaque mineralising treatments'.

Levels of Ca, Pi and F were increased in the plaque residue of samples taken from in situ devices following treatment three times daily for 3 days with two types of mineralising rinse [45]. An average Ca:Pi ratio of 1.52 strongly suggested that an apatitic phase formed after the test rinses. Elevation of Ca^{2+} ions in plaque fluid but not of ionic Pi, after a subsequent sucrose rinse, suggested release from plaque reservoir sites, such as bacterial cell walls, rather than precipitate dissolution. However, the authors noted that their plaque fluid samples were relatively highly supersaturated with respect to enamel, perhaps because they had only used a single 1-min rinse with 10% sucrose. Multiple sucrose rinses or prolonged sucrose exposure would have led to less saturated solution conditions, in which the mineral precipitate would be expected to play a more active enamel protective role. The authors cited a study by Cury et al. [50], in which levels of Ca, Pi and F in samples of plaque overlaying in situ enamel blocks decreased with increasing frequency of exposure to sucrose.

Vogel et al. [47] reported a 2-fold increase in plaque fluid Ca^{2+} ions after a sucrose rinse but a slight decrease in ionic Pi, irrespective of the prior administration of a high-F deposition rinse. These findings are consistent with those of Margolis and Moreno [4] noted in section 6.2. Vogel et al. [47] calculated that their plaque fluid samples were highly saturated with respect to fluorapatite 7 min after the sucrose rinse but, despite elevated free Ca, undersaturated with respect to dicalcium phosphate dihydrate and on the verge of undersaturation with respect to tooth enamel.

Augmentation of the Ca reservoir in plaque can also be provided by casein phosphopeptide preparations. Such materials, derived from milk, can bind amorphous CaPi and have been shown to inhibit demineralisation [51] and promote remineralisation [52] of enamel in situ in humans. Rose [53] recently demonstrated that the addition of casein phosphopeptide to a model bacterial plaque in vitro not only provided extra Ca^{2+}-binding sites but also restricted Ca diffusion through the plaque.

In their otherwise useful paper on the saturation of saliva with respect to Ca salts, Larsen and Pearce [54] make certain potentially misleading statements concerning CaF_2, in our opinion. These authors start their abstract with: 'It may be assumed that free ionic concentrations of Ca and phosphate in resting saliva tend to equilibrate with those in plaque fluid, and that salivary determinations can therefore be used to illustrate chemical conditions in both saliva and plaque.' and conclude with: 'Therefore, this salt (CaF_2, sic), the outcome of
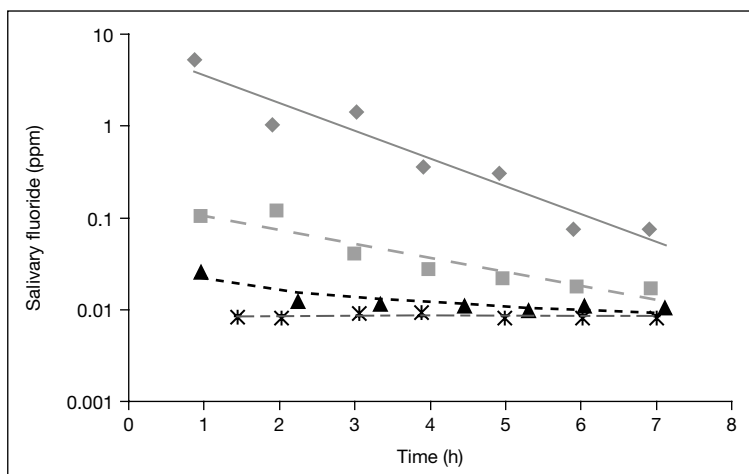
Fig. 3. Mean salivary fluoride concentration against time after rinsing for 1 min with various aqueous solutions (n = 8). ◆ = 1 ml 0.0526 mol/l CaCl$_2$ mixed with 1 ml 0.1052 mol/l NaF immediately before application; ■ = same solutions mixed 1.5 h before application; ▲ = 2 ml of equivalent concentration of CaF$_2$ as particle suspension aged for 3 months; ✳ = 2 ml 0.0526 mol/l NaCl control.

topical F therapy, will inevitably dissolve in the oral fluids.' Tatevossian's assertion [3], noted earlier, concerning the value of analyses of site-specific plaque samples over pooled plaque samples is even more pertinent when considering saliva. Whilst a reservoir of CaF$_2$ in plaque or elsewhere might be expected to dissolve on the basis of thermodynamics, according to the considerations of the above authors, the question of the rate of dissolution does not appear to have been addressed.

In an earlier study, Larsen and Ravnholt [55] showed that the dissolution kinetics of differently sourced CaF$_2$ and the 'calcium-fluoride-like' material that is actually formed in saliva (as opposed to in water) varied markedly, depending on both the different solid preparations and on the fluid (water or saliva). Saliva inhibits CaF$_2$'s precipitation and dissolution. This is illustrated in figure 3, which shows results of a salivary clearance study taken from a patent of Clarkson et al. [56]. Aqueous solutions of calcium chloride and NaF were mixed at various times relative to the application of the resultant 'CaF$_2$'-precipitating mixture to a group of individuals as a form of mouth rinse. Saliva samples were collected at least 1 h after the 1-min mouth rinse and were analysed for F after the addition of TISAB buffer, as described elsewhere [42]. The effect of the age of the precipitated material was dramatic: salivary F concentrations

measured in samples collected 1–3 h after mouth rinse use were on average ten times higher for freshly precipitated material than for material precipitated 1.5 h prior to use, which were, in turn, on average five times higher than a dispersion of 3-month-old CaF_2. Clarkson et al. [56] believed that their nascent CaF_2-like precipitate particles were stabilised by salivary orthophosphate, as described by others [57, 58]. Very recent work suggests that similarly stabilised particles can be created using monofluorophosphate [59].

The ability of tooth mineral itself to buffer pH, and hence restrict the cariogenic challenge from plaque acids, has been demonstrated in recent laboratory studies. Zaura et al. [60] showed that the pH at the bottom of in situ plaque, in grooves cut into different materials to simulate tooth fissures, decreased in the order: polyacrylate > enamel > dentine after an in vitro glucose treatment, i.e. the inverse of the solubility. In chapter 3, Lynch describes his own in vitro work in which the presence of dentine adjacent to a block of enamel could act as a sacrificial source of Ca and Pi and inhibit enamel dissolution in solutions initially undersaturated with respect to both materials.

6.5 Summary

One of the main conclusions in Chapter 1 was that oral Ca and Pi were the only salivary/plaque constituents that appeared to correlate with caries and calculus in the expected direction, in studies of many specific factors in isolation. In this chapter, where the focus has been on relatively recent investigations of plaque composition as a whole, plaque Ca is rarely a discriminating factor for caries. Measures of plaque acidity (pH and lactate) are more often statistically significant.

Further work is required in order to confirm the intuitively reasonable belief that a more composite measure, such as the DS of plaque fluid calculated by Margolis et al. and discussed in section 6.2, is a more applicable (inorganic) parameter for assessing, for example, caries risk. To date, little work has been done and those studies that have been reported have, for practical reasons, invariably used small numbers of subjects and often pooled plaque samples. The availability of semi-automated micro-analytical techniques, such as capillary electrophoresis, and the development of micro-electrodes, for example, those of Vogel et al., should enable researchers to analyse site-specific samples more easily.

The predominantly theoretical work of Dawes and Dibdin has drawn attention to the potential importance of the kinetics of ion and molecular transport through plaque in the caries process. Only very recently, with the application of micro-dissection methods [40, 41] and instrumental techniques such as

confocal microscopy [61, 62] and two-photon fluorescence imaging [63, 64], can the actual structure of plaque be investigated and associated studies of molecular transport through 'natural' plaque be made.

In many instances, the influence of F may overshadow those of the 'native' inorganic plaque components when assessing caries risk. A common finding in the studies described in section 6.3 is the slow clearance of F from plaque, following topical application, compared to clearance from saliva. A key finding is that F is not evenly distributed around the mouth. Not surprisingly, F concentrations tend to be higher at salivary stagnation sites and in plaque adjacent to those sites. In terms of caries control, of course, those same 'reservoir' sites also tend to favour retention of cariogenic substances, such as sucrose, and the relatively slow salivary clearance of subsequently generated plaque acids. The current state of knowledge is insufficient to estimate with certainty the ability of saliva to act as a conduit for F transfer from the main F reservoir in the mouth, the oral soft tissues, to the site of action: the plaque–tooth interface. A theoretical model based on chemical engineering principles of mass transfer could be valuable.

Acknowledgements

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