ADHERENCE, AGGREGATION AND HYDROPHOBICITY OF ORAL BACTERIA

with particular reference to microorganisms implicated in periodontal disease

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DECLARATION

This thesis is the original work of the author.

SUMMARY

There is little information concerning the factors involved in the colonization of the human mouth by bacteria implicated in the aetiology of periodontal disease. Therefore, the present studies were undertaken firstly to develop suitable assay procedures and secondly to employ these methods to investigate the colonization of the mouth by such bacteria.

The bacteria used in this study included species believed to be involved in the pathogenesis of periodontal disease, namely, Bacteroides gingivalis, Bacteroides intermedius, Capnocytophaga species, Haemophilus actinomycetemcomitans, Haemophilus aphrophilus and Peptostreptococcus species, as well as species representative of the normal commensal flora, namely, Veillonella species, Actinomyces israelii, Streptococcus salivarius and Streptococcus sanquis. Most research groups have studied type cultures because they are well characterized and freely available. However, it is known that repeated subculture on laboratory media can induce changes in bacterial surface components which may alter adherence. Therefore, a freshly isolated strain and a type culture were selected for each species studied (with the exception of the two Haemophilus species) giving a total of eighteen test bacteria.

The adherence of the 18 test bacteria to exfoliated buccal epithelial cells in vitro was studied with an epifluorescent staining technique using acridine orange in an acetate/NaOH buffer and potassium permanganate. Approximately half of the strains tested

adhered well to buccal cells, although adherence did not correlate in all cases with the generally accepted *in vivo* distribution of the tested species on the buccal mucosa. Mainly, the fresh strains adhered better than the type cultures. Of the Gram-negative bacilli studied, only the fresh isolate of *B. gingivalis* adhered well, as did the fresh and type *Peptostreptococcus* and *Veillonella* strains and the fresh *Streptococcus* strains.

Since the use of buccal cells presents several problems, such as contamination with indigenous bacteria, an adherence assay using tissue culture cells (HeLa) was developed. Monolayers of HeLa cells on glass coverslips were treated with saliva or serum prior to performing the adhesion assays to mimic the buccal or crevicular environments, respectively. As with the buccal cells, most of the fresh strains adhered better than the type strains, regardless of the treatment of the Hela cells. Generally, the results obtained for the buccal cells and saliva treated HeLa cells were similar. The bacteria usually associated with the gingival crevice tended to adhere poorly to saliva treated HeLa cells, but when HeLa cells were treated with serum, some of these bacteria adhered slightly better. Thus salivary and crevicular components appear to be specifically implicated in the selective adherence and possibly the colonization of bacteria on oral mucosal surfaces.

The other main surface available in the mouth for microbial colonization is the tooth surface. To mimic the *in vivo* situation, an assay was developed to study bacterial adherence to sections of natural tooth enamel pre-treated with saliva. Of the Gram-negative bacilli studied, the fresh *B. gingivalis* and *B. intermedius* strains

and the type H. actinomycetemcomitans strain adhered well, as did the type Peptostreptococcus species and both Veillonella strains. Neither of the A. israelii strains adhered well, and of the Streptococcus species, only the fresh S. sanguis adhered in high numbers. Generally, the fresh strains adhered best, as with epithelial cells.

In addition to mucosal and tooth surfaces, bacteria may also adhere to pre-existing bacterial accumulations on these surfaces. Such interactions were studied by measuring the resultant aggregation of single bacterial suspensions mixed with saliva (homotypic aggregation) and of mixed suspensions of different strains suspended in buffer (heterotypic aggregation) using an accurate spectrophotometric method. In the homotypic aggregation assay, approximately half of the strains aggregated in saliva. The heterotypic aggregation assay revealed two main groups of aggregating bacteria: (i) B. gingivalis and B. intermedius isolates aggregated with the S. salivarius, S. sanguis and A. israelii strains; and (ii) B. gingivalis, B. intermedius and Capnocytophaga strains aggregated with one another. There was no heterotypic aggregation among any of the Gram-positive species. Also, no correlation existed between bacterial aggregation and adherence to the surfaces tested.

Since hydrophobicity is believed to be important in microbial adherence, the hydrophobic potentials of the test bacteria were investigated by assessing their adherence to xylene. The hydrophobic potentials of the bacteria were found to be markedly modified by the composition of the suspending medium, and by the addition of saliva. Significant correlations were evident between bacterial hydrophobicity and adherence to surfaces exposed to saliva, but there were no

significant correlations with homotypic or heterotypic aggregation.

The results from this study indicate that bacterial adherence to the different oral and bacterial surfaces tested involves specific interactions, although adherence may be enhanced by non-specific hydrophobic interactions. The freshly isolated strains adhered in higher numbers than the type cultures, with the exception of the Peptostreptococcus species which gave the converse result. This trend was evident regardless of the surface tested, suggesting that the loss of adherence confering components by laboratory maintained bacteria is of a generalized nature, as opposed to the loss of specific binding sites. Contrary to the above findings, no differences were noted between the type and fresh strains in the aggregation or hydrophobicity assays, indicating that components which confer adherence are not necessarily involved in these other interactions.

A variety of factors appear to be important in the colonization of the oral cavity by different bacteria implicated in periodontal disease. Generally, the Bacteroides and Capnocytophaga species adhered poorly to the different surfaces tested, although the Bacteroides and Capnocytophaga strains aggregated with each other and with the Streptococcus strains. Bacteroides may therefore adhere to pre-existing supragingival dental plaque containing streptococci, and to subgingival plaque dominated by Gram-negative bacteria. In addition, aggregation of the Bacteroides and Capnocytophaga species may aid in the retention of these bacteria in the gingival crevice. In contrast, the Haemophilus and Peptostreptococcus strains generally adhered in high numbers to the test surfaces but demonstrated little heterotypic aggregation.

ABBREVIATIONS

ABB	anaerobe blood broth
IU	international units
LTA	lipotechoic acid
ND	no data available
NS	not significant
p	probability
PBS	phosphate buffered saline
rpm	revolutions per minute
SEM	standard error of the mean
SIB	saliva ions buffer
TSB	tryptic soy broth
v/v	volume to volume

CHAPTER 1

LITERATURE REVIEW

1.1 THE ORAL ENVIRONMENT

The mouth consists, not of one uniform surface, but of several distinct habitats, each supporting a characteristic microbial flora which is determined by the physical, chemical and biological properties of the tissues and secretions in that site (Gibbons, 1984). The various habitats available for growth include the buccal mucosa, tongue, hard and soft palate, gingivae, gingival crevice, teeth and saliva, with the largest accumulations of bacteria found on teeth and on the dorsum of the tongue. Teeth are the only hard non-shedding surfaces available for microbial colonization in the body, and the dental plaques which form on these surfaces have been extensively studied due to their causal relationship with dental caries and periodontal disease. On mucosal surfaces, epithelial cell desquamation prevents the formation of plaques as occurs on teeth, and as a result a different microflora is found (Gibbons and van Houte, 1975).

Dental plaque consists of densely packed bacteria embedded in an amorphous material consisting predominantly of extracellular polymers synthesized by bacteria and of macromolecules derived from saliva, crevicular fluid and dietary sources (Fitzgerald, 1985). Between the surface of the tooth enamel and the plaque is the acquired pellicle, consisting of salivary glycoproteins which are selectively adsorbed to

the enamel surface (Ericson et al., 1982). Adsorption of salivary components to clean tooth surfaces occurs within minutes (Ericson et al 1982) and bacterial aggregates soon follow. The aggregates gradually increase in size and eventually coalesce to form a continual bacterial layer which increases in thickness until limited by abrasive forces (Bjorn and Carlsson, 1964). A single tooth may, however, present several distinct surfaces, each with a specific population of bacteria. For example, areas between adjacent teeth and in the gingival crevice offer protection from the adverse conditions an organism would be subjected to on an exposed occlusal surface (Osterberg, Sudo and Folke, 1976).

The mouth is continually bathed in saliva, which is produced by the parotid, submandibular, sublingual and minor salivary glands. Whole saliva contains several ions including sodium, potassium, chloride, calcium, magnessium, phosphate and bicarbonate. The major organic constituents of saliva include mucins, immunoglobulins, lysozyme and amylase (Mason and Chisholm, 1975). Saliva is thought to protect mucosal surfaces from dessication and to act as a lubricant (Tabak et al., 1982) as well as modulating bacterial colonization by its mechanical washing action and by affecting the aggregation and adherence of bacteria (Stinson et al., 1982). Saliva may also affect colonization because it is likely (due to its composition and continuous production) that it is the main source of nutrients for bacteria in the mouth (van der Hoeven et al., 1984).

In addition, the oral cavity is supplied with crevicular fluid via the gingival crevice, which is also thought to have a protective function due to its flushing action and the presence of immune system

components. The composition of crevicular fluid resembles serum, containing albumin, fibrinogen, lysozyme, immunoglobulins, complement components, neutrophils, lymphocytes and monocytes (Cimasoni, 1983). Crevicular fluid may also provide essential growth factors for some populations of bacteria; for example Bacteroides melaninogenicus may acquire haemin (van Palenstein Helderman, 1981a).

A wide variety of bacterial types are indigenous to the oral cavity and many workers have investigated the proportions of bacteria characteristically present on various oral surfaces and in saliva. Table 1.1 illustrates this diversity. Streptococci and filamentous organisms comprise major segments of the flora present on most sites, although Streptococcus salivarius preferentially colonizes the dorsal surface of the tongue, whereas S. sanguis and S. mutans have the teeth as their preferred habitat. Spirochetes, and Bacteroides species primarily colonize the gingival crevice area, which constitutes a highly reduced area with Eh levels to -150 mV (Kenney and Ash, 1969).

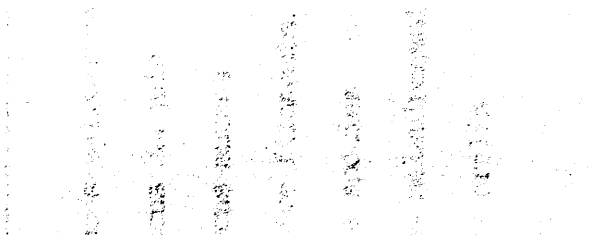


Table 1.1 Approximate percentage distribution of bacteria on various oral surfaces and in saliva (after Gibbons and van Houte, 1975).

Bacteria	subgingival plaque	supragingival plaque	tongue dorsum	buccal mucosa	saliva
Streptococcus salivarius	<0.05	<0.05	20	11	20
Streptococcus sanguis	8	15	4	11	8
Streptococcus mutans	-	0-50	<1	<1	<1
Gram-positive filaments	35	42	20	-	15
Veillonella species	10	2	12	1	10
Bacteroides melaninogenicus	6	<1	<1	<1	<1
Bacteroides oralis	5	5	4	-	-
Spirochetes	2	<0.1	<0.1	<0.1	<0.1

The data are approximate percentages of the total isolates recovered on blood agar incubated anaerobically.

1.2 BACTERIAL ADHERENCE

1.2.1 Adherence and colonization

The microbial populations of various sites within the oral cavity vary qualitatively and quantitatively, for example the microbial flora of the gingival crevice is markedly different from that of the dorsum of the tongue (Gibbons and van Houte, 1975). This wide variance is due to a number of selective pressures acting at any one site thereby producing a distinct population. For instance, the mechanical washing action of saliva is an important factor in limiting microbial colonization of the mouth (van Houte, 1983). To colonize succesfully, bacteria must either multiply at a rate exceeding the dilution rate caused by the flow of saliva, or adhere to and proliferate on one of a number of oral surfaces. Evidence suggests that the rate of multiplication of bacteria colonizing oral surfaces is low, averaging only two to four divisions each day (Gibbons, 1984), and during waking hours bacteria are removed about every four minutes by swallowing saliva. Therefore, it is unlikely that unattached bacteria would be able to maintain themselves solely by multiplying in saliva.

The influence of the adhesive propeties of oral bacteria on their intra-oral distribution was first suggested by van Houte, Gibbons and Banghart in 1970. They investigated the ability of *Streptococcus* species to adhere to human tooth enamel powder *in vitro*, and to the tooth surface *in vivo*. They concluded that the observed differences in the proportions of *S. salivarius* and *S. sanguis* on tooth sufaces was due, not only to the ability of these bacteria to

grow in that environment, but more importantly to their ability to adhere to the tooth surface.

During further investigations by Gibbons and van Houte (1971) the relationship between the adherence of Streptococcus species to oral epithelial cells and the natural distribution of these bacteria to epithelial surfaces within the oral cavity, was studied. They reported that S. salivarius and S. sanguis, which are present in significant proportions on epithelial cells in vivo, were found to possess a definite capacity to adhere to buccal epithelial cells in vitro. In contrast, S. mutans which is rarely found on epithelial surfaces, exhibited weak or no adherence to buccal epithelial cells in vitro. In addition, mixtures of streptomycin-resistant labelled streptococci were introduced into the mouths of volunteers. Subsequently, labelled S. salivarius and S. sanguis were recovered in high proportions from cheek and tongue surfaces, whereas labelled S. mutans were recovered from these surfaces in comparatively low numbers.

A number of other studies have subsequently produced similar conclusions on the importance of adherence in colonizing the oral cavity. For example, Weerkamp and McBride (1980b) used two strains of S. salivarius; a wild-type (HB) and a mutant (HB-7) which had lost its ability to aggregate in saliva or to adhere to buccal cells. Six volunteers were inoculated with a mixture of strains of HB and HB-7. After an hour, more HB were found on buccal mucosa and cleaned tooth surfaces than HB-7. Eventually HB-7 was cleared from the mouth, whereas HB remained for up to three months. This indicates that a

bacterium which lacks the ability to adhere to oral surfaces is rapidly lost from the mouth *in vivo*.

Liljemark and Gibbons (1971) found that *Veillonella* and *Neisseria* species adhered poorly to pre-formed dental plaques, and also that *Neisseria* species adhered poorly to the tongue dorsum, although the *Veillonella* species adhered well to this surface. The intra-oral distribution of these organisms correlated with these findings in that the *Veillonella* were found in high proportions on the tongue and in low numbers on pre-formed dental plaque, and the *Neisseria* were found in low proportions on both surfaces. It was concluded that the adherence of these bacteria to oral surfaces determined their proportions found indigenously.

Wheeler, Clark and Birdsell (1979) reported that *Actinomyces viscosus* adhered well to saliva treated hydroxyapatite *in vitro*, which correlated with *in vivo* experiments using streptomycin-resistant strains introduced into the mouths of volunteers. Qureshi and Gibbons (1981) assessed the adherence of *A. viscosus* to hydroxyapatite treated with saliva from donors of different ages. Strains of *A. viscosus* were found to adhere better to hydroxyapatite treated with saliva from older children and adults than from younger children. They proposed that changes in the composition of saliva corresponding to the age of the individual accounted for the increased frequency of *A. viscosus* with age in children. In contrast, *A. naeslundii* and *S. mutans* were unaffected by the age of the saliva donor and showed no age dependent colonization patterns.

Brecher, van Houte and Hammond (1978) studied the oral colonization of rats by two strains of A. viscosus. The first, a virulent strain was capable of colonizing fissures and tooth surfaces near the gingival margin, and of causing alveolar bone loss. The second strain was avirulent and could only colonize tooth fissures. It was suggested that the virulence of the first strain was related to its ability to form plaque on smooth tooth surfaces near the gingiva, which correlated with its ability to adhere well to hydroxyapatite in vitro compared with the avirulent strain.

It is evident that the degree to which a bacterium may attach to a surface exposed to the flow of saliva influences the extent to which it may colonize. Therefore, the ability of a bacterium to adhere to oral tissues may be considered to be a necessary selective requirement. Interfering with the adherence factors on bacterial or host surfaces may influence the ability of a bacterium to adhere. The extent of inhibition may be sufficient to result in the eventual elimination of that organism from the oral cavity and may provide new ways of controlling infections.

1.2.2 Effect of bacterial concentrations

Generally, the extent of bacterial colonization of oral surfaces is influenced not only by the innate capacity of bacteria to adhere, but also by the number of cells available. In vitro studies have shown that the number of bacteria that adhere to hydroxyapatite or buccal cells is directly related to the initial bacterial concentration within a range of approximately 10^7 to 10^9 bacteria per millilitre (Hartley, Robbins and Richmond, 1978; Eifert, Rosan and

Golub, 1984). Similarly, *in vivo* experiments by van Houte and Green (1974) demonstrated that the number of bacterial cells recoverable from a tooth surface shortly after cleaning is related to the concentration of the species in saliva. Below a certain salivary concentration, no organisms can be recovered from the teeth. In the case of *S. sanguis* this critical concentration is approximately 10^3 to 10^4 bacteria per ml of saliva, whereas with *S. mutans* the minimum salivary concentration is about ten-fold higher due to this organisms innately lower affinity to adhere to tooth surfaces.

At low bacterial concentrations the numbers of bacteria that may subsequently adhere to a surface do not necessarily follow a linear relationship. Gibbons, Moreno and Etherden (1983c) performed adherence experiments with saliva treated hydroxyapatite and different concentrations of *S. sanguis*. They showed that the use of low bacterial concentrations (approximately 10^7 bacteria per ml) produced results that indicated low numbers of binding sites were available on the hydroxyapatite which had high affinities for *S. sanguis*, whereas high bacterial concentrations (approximately 10^9 bacteria per ml) indicated larger numbers of binding sites, but with much lower affinities. It was concluded that there are multiple binding sites for *S. sanguis* on saliva treated hydroxyapatite. At low concentrations, bacteria adhere to the more specific high affinity sites that become saturated when higher concentrations are used. More recently, Gibbons, Etherden and Moreno (1985a) reported that the high affinity binding site of *S. sanguis* involved stereochemical interactions between a cell surface lectin and a sialic acid residue on saliva coated hydroxyapatite. The affinity of this site was

approximately 400 times that of the less specific hydrophobic-electrostatic interaction sites that predominate at high bacterial concentrations. The data given represented a typical two-site adsorption model. However, treatment of the saliva treated hydroxyapatite with neuraminidase, which destroys the sialic acid receptors, gave a typical one-site adsorption model; as did the use of untreated or albumin treated hydroxyapatite. These phenomena were also studied by Clark, Bammann and Gibbons (1978) who suggested that, in general, at high bacterial concentrations the number of binding sites will correlate better with the number of bacteria that adhere, whereas at low concentrations the effect of affinity will be more apparent.

Gibbons (1984) suggested that when an infective agent gains access to the oral cavity, the small numbers involved bind to high affinity sites. As the organism proliferates and the infection proceeds, the increased numbers of bacteria saturate the high affinity sites and bind to the low affinity sites. Finally, as the bacterial population increases, further colonization is limited as the remaining sites have too low an affinity to permit attachment. Although these studies were all performed with S. sanguis adhering to saliva treated hydroxyapatite, it is possible that similar multiple binding sites may exist on tooth enamel or epithelial cells for other bacterial species.

1.2.3 Adherence interactions

Appelbaum et al. (1979) used radio-labelled S. sanguis to study the effect of competing bacterial species in adhesion assays. Strains of similar sero-types of S. sanguis were found to compete for binding

sites, whereas S. salivarius, S. faecalis, S. mitior and Capnocytophaga species showed little or no competition. Also, strains of S. mutans and S. sobrinus (formerly S. mutans serotypes d, g and h) adhered to different receptors in salivary pellicles on hydroxyapatite (Gibbons, Cohen and Hay, 1986). Similarly, Kagermeier and London (1985) found that two strains of Haemophilus actinomycetemcomitans adhered to different binding sites on hydroxyapatite. Thus it seems that different bacterial species may attach to quite different receptor sites on oral surfaces. Furthermore, the analysis of adherence data indicates that different numbers of binding sites exist for different bacterial species (Clark et al., 1978; Gibbons et al., 1983c).

The number of binding sites present on the bacterial surface may also vary. Morris and McBride (1984) indicated that S. sanquis possessed two different binding sites mediating its attachment to different receptors in salivary pellicles on hydroxyapatite. Also, Weerkamp and Jacobs (1982) isolated and purified three cell wall associated protein antigens from S. salivarius. One involved in adherence to erythrocytes, one associated with aggregation with Veillonella species, and the third appeared not to be linked to any established adhesion function. These studies also showed that S. sanquis and S. salivarius may lose some of these factors, thus producing adherence deficient mutants.

Adherence studies with S. sanquis and saliva treated hydroxyapatite have shown that productive interactions between a bacterium and the substrate surface may occur which can enhance the probability of other bacteria subsequently adhering (Nesbitt et al.,

1982b). This has been termed positive cooperativity, and mechanistically, there are several means by which this can occur. For example, one bacterium in a chain may initially adhere bringing the rest close enough to also initiate adherence. However, Nesbitt et al. (1982b) presented data indicating that this theoretical event did not appear to be important in explaining the results they obtained. They suggested that, on a molecular basis, when one bond such as a hydrogen bond is formed, adjacent bonds may be stabilized. Also, if hydrophobic forces are involved, the formation of hydrogen or ionic bonds may exclude competing ions or water molecules in the near vicinity and so favour hydrophobic interactions. The net effect of such events would be the promotion of subsequent adherence interactions following the initial reaction.

1.2.4 Bacterial surface structures

Adherence interactions must involve complimentary components on the colonizable host surface and on the bacterial cell surface. Bacterial surface structures have been shown to vary widely depending on the species or even strain of bacterium studied (Slots and Genco, 1984; Handley et al., 1985) and these may influence the specificity noted with bacterial adherence (Gibbons and van Houte, 1975).

Black pigmented Bacteroides species have been shown, using electron microscopy, to possess fimbriae, although the different species may have various types (Handley and Tipler, 1986). The function of the different types of fimbriae is not clear, but they appear to be involved in the mediation of adherence (Slots and Gibbons, 1978; Okuda, Slots and Genco, 1981). Oral Bacteroides

species have also been shown to possess long fibres distinct from fimbriae, as well as capsules (Woo, Holt and Leadbetter, 1979).

The Capnocytophaga species (Leadbetter, Holt and Socransky, 1979) have been shown to possess long fibrils, vesicles and extracellular amorphous material (Holt, Leadbetter and Socransky, 1979; Poirier, Tonelli and Holt, 1979). Also, Haemophilus actinomycetemcomitans was reported to have thin surface projections, capsules and an amorphous surface material (Holt, Tanner and Socransky, 1980). However, despite the morphological data available on the surface structures of these organisms, their role in adherence is unclear.

Electron microscopic studies have also shown that S. salivarius and S. sanguis possess two distinct types of surface structures, namely fimbriae (less than 20 μm in length) and fibrils (0.15 to 0.46 μm in length) (Handley, Carter and Fielding, 1984; Handley, et al., 1985). Furthermore, these surface structures have been shown to mediate adherence to oral surfaces (Gibbons, Etherden and Skobe, 1983b; Weerkamp, van der Mei and Liem, 1984; Robinson and Handley, 1984). Also, Shaw, Swindin and Leach (1985) reported that a number of strains of streptococci that had lost their fimbriae on repeated sub-culturing in vitro, regained their fimbriae when grown in sterile saliva and may therefore regain their adhesive properties in vivo.

Electron microscopic studies of Actinomyces species have shown a wide variability in the fimbriae on different strains. This variation was reported to not always correlate with adherence, but strains with few or no fimbriae generally adhered poorly (Clark et al., 1981).

Ellen, Walker and Chan (1978) reported that the removal of such fimbriae by homogenization inhibited aggregation and adherence. More recently, two types of fimbriae were identified on A. viscosus and A. naeslundii using anti-fibril antibodies, but it was found that only one type was involved in adherence. Furthermore, the different abilities of these species to colonize tooth and epithelial surfaces appears to be associated with the distribution of these fimbriae (Cisar, Sandberg and Mergenhagen, 1984; Clark, Wheeler and Cisar, 1984; Clark et al., 1986). Analysis of Actinomyces fimbriae indicated that protein is the major component of these structures (Masuda et al., 1983).

1.2.5 Salivary mucins

It is clear that the adherence of microorganisms to a surface will be influenced by the suspending medium. In the oral cavity this is saliva, which contains a complex mixture of components that may adsorb to and modify the surfaces of colonizing bacteria or of the host tissues.

Salivary mucins are considered to play a central role in modulating bacterial adherence. Mucins are high molecular weight glycoproteins containing approximately three per cent N-acetyl neuraminic acid (sialic acid) (Hay, Gibbons and Spinell, 1971; Tabak et al., 1982). There is evidence that these glycoproteins can interact with specific bacterial surface components and can selectively adsorb to hydroxyapatite, thereby contributing to the acquired enamel pellicle. The sialic acid of mucins adsorb strongly to hydroxyapatite (Ericson, 1967) forming binding sites specific for

certain bacteria (Gibbons et al., 1985a), the adherence of which will be inhibited by the removal of sialic acid from the pellicle (Stinson et al., 1982). It has been suggested that the differing abilities of such salivary constituents to interact with various bacteria may determine the specifities observed in the adherence of bacteria to teeth (Hay et al., 1971; Stinson, Jinks and Merrick, 1981).

Stinson et al. (1982) showed that although the selective deposition of mucin on oral surfaces promotes the adherence of S. sanguis and S. mutans, mucin mediated aggregation of these bacteria may also play a role in their clearance from the oral cavity. Gahnberg et al. (1982) chromatographically fractionated saliva and reported that high molecular weight salivary components promoted the adherence of S. mutans to hydroxyapatite, while other salivary fractions inhibited adherence. It was concluded that the influence of saliva on adherence is dependent on the net effect of adherence promoting and adherence inhibiting factors.

1.2.6 Fibronectin

Fibronectin is a large glycoprotein found in serum, saliva and salivary components adsorbed to epithelial cells and hydroxyapatite (Ruoslahti, Engvall and Hayman, 1981; Babu et al., 1983). Studies have shown that fibronectin can also bind to a variety of bacteria and may markedly influence their adherence to epithelial cells and to saliva treated hydroxyapatite (Simpson and Beachey, 1983; Babu et al., 1983; Kuusela et al., 1985; Simpson, Hasty and Beachey, 1985; Stanislawski et al., 1985; Babu and Dabbous, 1986; Courtney et al., 1986). S. sanguis, S. salivarius, S. mutans and S. pyogenes have been

reported to bind fibronectin avidly, whilst B. gingivalis, A. viscosus, A. naeslundii and Escherichia coli bind only small amounts (Babu et al., 1983; Imai et al., 1984; Courtney et al., 1986; Ericson and Tynelius-Bratthall, 1986).

Fibronectin has been suggested as a receptor on oral epithelial cells for lipotechoic acid on the surface of group A streptococci (Simpson and Beachey, 1983; Courtney, Simpson and Beachey, 1983; Courtney et al., 1985 and 1986). In addition, fibronectin adsorbed to buccal cells or tissue culture cells has been reported to promote the adherence of S. pyogenes, but inhibit the adherence of E. coli (Stanislawski et al., 1985). Exogenously acquired fibronectin may therefore mediate bacterial adherence by providing attachment sites for some bacteria and inhibiting the adherence of other bacteria.

Studies have also indicated that the amounts of fibronectin adsorbed to epithelial cells, obtained by scraping the buccal mucosa, varied considerably. This is probably dependent on the degree of exposure of the cells to saliva in situ (Abraham, Beachey and Simpson, 1983; Simpson et al., 1985).

1.2.7 Immunoglobulin A

Salivary immunoglobulin A antibodies (IgA) have been widely implicated in the adherence and colonization of oral bacteria (Cole, 1985). IgA has been shown to comprise approximately two per cent of the dry weight of human dental plaque and has been found in the salivary pellicle in considerable quantities (Taubman, 1974). Kilian, Rolland and Mestecky (1981) found that IgA could bind to

hydroxyapatite and increase the adherence of S. mutans (grown without sucrose), S. salivarius and S. mitior, and that IgA mixed with these bacteria inhibited their subsequent adherence. However, no effect was noted with A. viscosus, A. naeslundii or sucrose grown S. mutans.

Tomasi (1972) reported that IgA can bind specifically to antigenic components on the bacterial surface, which may affect adherence. Furthermore, salivary antibodies appear to be synthesized locally in the salivary glands, as they will react with oral strains of bacteria, but not with intestinal strains of bacteria (Sirisinha, 1970). Gibbons and van Houte (1975) suggested that IgA mediated immunity is developed against many or all oral bacteria, and particular serotypes can only colonize a host for finite periods of time. It was postulated that such serotypes will eventually be displaced by different strains which will be produced either by mutation or from exogenous sources.

1.2.8 Enzymic modification of salivary components

Salivary components involved in adherence reactions may be susceptible to modification by enzymes derived from oral bacteria. Gibbons and Etherden (1982) reported that saliva that had been incubated at 35°C for 18 hours, to promote the elaboration of microbial enzymes, formed pellicles which exhibited markedly different adherence properties. Exposure of saliva treated hydroxyapatite to purified enzymes, similarly modified adherence properties. In addition, Sato, Koga and Inoue (1983) demonstrated that enzymes derived from plaque bacteria inhibited the adherence promoting functions of salivary proteins for S. sanguis. More specifically,

Wirstrom and Linde (1986) noted that fibronectin adsorbed to polystyrene could be degraded by various oral bacteria, which would presumably affect subsequent bacterial adhesion.

1.2.9 Bacterial lipotechoic acids

Lipotechoic acid (LTA) is an amphipathic molecule composed of 1,3 phosphodiester-linked glycerophosphate and a small lipid moiety (Wicken and Knox, 1975). LTA is found in most, but not all, Gram-positive bacteria. Exceptions lacking LTA include the genera Actinomyces (Wicken et al., 1978) and Micrococcus (Owens and Salton, 1975), and strains of S. mitior (Rosan, 1978). LTA is located mainly in the bacterial cell wall, but may also be actively excreted into the surrounding medium (Joseph and Shockman, 1975; Markham et al., 1975).

LTA has been implicated in the adherence of Streptococcus species, mainly S. pyogenes. The role of LTA in the adherence of streptococci was indicated by the inhibition of adherence noted when skin or buccal epithelial cells were pre-treated with LTA from S. pyogenes (Alkan, Ofek and Beachey, 1977; Beachey, 1975). However, it was argued by Wicken (1980) that this could be due to LTA binding to the human cells giving them a considerable negative charge, causing the subsequent repulsion of similarly charged bacterial cells. Bolton (1980) obtained antisera from rabbits to the techoic acids of Bacillus species. The antiserum inhibited the adherence of radiolabelled S. mutans, S. sanguis and Bacillus species to hydroxyapatite and saliva treated hydroxyapatite and therefore supports the role of LTA in streptococcal adherence. However, Stashenko et al. (1986) have reported different findings. Using monoclonal antibodies against

S. mutans LTA, they found that concentrations of monoclonal antibodies between 0.3 and 3.0 μg/ml had no effect on the initial adherence of S. mutans to saliva treated hydroxyapatite or on subsequent plaque accumulation. Furthermore, an increased concentration of 30 μg/ml enhanced adhesion. It was therefore concluded that LTA does not play a major role in S. mutans adherence or plaque formation.

Miorner, Johansson and Kronvall (1983) found a correlation between the LTA content of the surface of group A streptococci and the surface hydrophobicity of the bacteria. It was concluded that LTA is the major component determining the surface hydrophobicity of group A streptococci, which may influence their adherence. Rolla, Iverson and Bonesvoll (1978) suggested that LTA, which will contribute to the negative charge of bacteria, may be the component responsible for the binding of streptococci to teeth via electrostatic forces. However, generalized hydrophobic and electrostatic interactions do not explain the specificity of attachment noted with some bacteria.

S. pyogenes strains isolated from the skin have different adherence properties to those isolated from the throat, although LTA derived from these strains has similar adsorption properties (Alkan et al., 1977). Similarly, S. salivarius and S. mutans, which possess LTA (Wicken and Knox, 1975), adhere poorly to saliva treated hydroxyapatite (Gibbons, 1984), whereas S. mitior (Rosan, 1978) and A. viscosus (Hamada, Tai and Slade, 1976; Wicken et al., 1978) do not contain detectable levels of LTA, but adhere in high numbers to saliva treated hydroxyapatite (Gibbons, 1984; Qureshi and Gibbons, 1981). These studies indicate that if LTA is involved in adherence, it is more likely to mediate non-specific adherence interactions.

However, interactions of a specific nature between LTA and oral surfaces have been alluded to by Simpson and Beachey (1983) who suggested that fibronectin on oral epithelial cells could serve as a receptor for the LTA of S. pyogenes and thereby facilitate its adhesion. This was confirmed by Courtney et al. (1986) who found that antibodies to fibronectin inhibited the adherence of S. pyogenes to buccal cells. Courtney et al. (1983) suggested that human plasma fibronectin contains binding sites specific for fatty acids and that streptococcal LTA binds to these sites by its glycolipid moiety. More recently it was shown that fibronectin will bind to S. pyogenes and inhibit its adherence to hexadecane, and also that this inhibitory activity was prevented by pre-treating the fibronectin with LTA (Courtney et al., 1985). Fibronectin therefore contains at least one population of high affinity binding sites for LTA.

Therefore, overall a large amount of data is available that associates LTA with adherence, however a number of reports provide evidence to the contrary. It may therefore be concluded that the role of LTA in adherence is unclear and requires further work.

1.2.10 Lectins

The adherence of oral bacteria may be influenced by lectin like adhesins. The adherence of S. mutans (Gibbons and Qureshi, 1979) and S. sanguis (Nagota et al., 1983) has been shown to be inhibited by galactose. It was suggested that these organisms may possess lectins that interact with the saccharide receptors of salivary glycoproteins adsorbed to oral surfaces. Also, Murray et al. (1982) isolated a sialic acid binding lectin from S. sanguis and S. mitior that

interacts with the sialic acid of salivary mucins adsorbed to hydroxyapatite.

It has also been reported that lectins can be adsorbed onto oral surfaces from certain foods. Buccal epithelial cells, collected before and after eating wheat germ, were tested for their ability to adsorb S. sanquis. Buccal cells collected after eating wheat germ were subsequently found to adsorb more bacteria (Gibbons and Dankers, 1983).

1.2.11 Adherence of bacteria implicated in periodontal disease

The connection between adherence and colonization is thus well documented and various factors involved have been studied. However, the data available relate mainly to streptococci and little work has been done with the bacteria thought to be implicated in periodontal disease. Streptococci have been more widely studied probably because of their aetiological role in dental caries and for technical reasons associated with the adherence assays in general use. For instance, streptococci can be isolated and grown easily, and are comparitively large cells that stain well with conventional stains such as Gram's making them suitable for use with microscopic techniques. Conversely, most of the bacteria implicated in periodontal disease are small and stain poorly. Consequently, little is known about the importance of adherence for these bacteria to different oral surfaces, or to which surfaces they preferentially adhere.

1.3 BACTERIAL AGGREGATION

1.3.1 Aggregation and colonization

Bacteria already established on epithelial or tooth surfaces provide another important and variable surface to which other bacteria may adhere. Bacteria of the same or different species may adhere to each other, referred to as homotypic and heterotypic aggregation, respectively. Such interactions are believed to help in the sequential colonization of the oral cavity and also to protect the host from exogenous bacteria (Gibbons and van Houte, 1975).

In relation to the latter function, the aggregation of bacteria within the mouth has been shown to be a host defence mechanism for the clearance of bacteria (Stinson et al., 1982). Salivary glycoproteins were shown to inhibit the attachment of S. salivarius, S. sanguis and S. mitior to human buccal epithelial cells and to foster the desorption of previously attached bacteria. The properties of the glycoproteins were found to correlate with their ability to aggregate the streptococci (Williams and Gibbons, 1975). Liljemark, Bloomquist and Germaine (1981) demonstrated that the formation of large aggregates in adherence experiments resulted in a decrease in the numbers of organisms which attached, although the formation of small aggregates caused an increase in adherence. They suggested that as bacteria aggregate, the increase in total particulate mass reduces the likelihood that aggregates can adhere or remain adherent due to physical forces such as the flow of saliva, hence resulting in bacterial clearance.

In contrast, bacterial aggregation has also been shown to promote adherence. McBride and van der Hoeven (1981) took rats with established S. mutans plaques and inoculated them with two strains of Veillonella; one strain that could aggregate with S. mutans, and an aggregation deficient mutant strain. The wild-type was able to colonize, but the mutant strain was eliminated. A similar study was carried out by Weerkamp and McBride (1980b) in vivo with humans using a wild-type and a mutant strain of S. salivarius. The mutant had lost the ability to aggregate homotypically in the presence of saliva or to adhere to buccal epithelial cells, but could still aggregate with Veillonella and Fusobacterium species. Volunteers were inoculated with mixtures of the wild-type and mutant strains. Subsequently, large numbers of the wild-type strain were found on the buccal mucosa and clean tooth surfaces compared to the mutant strain. However, similar numbers of the test bacteria were found on the tongue where Veillonella and Fusobacterium species were present naturally in high numbers (Weerkamp and McBride, 1980b).

Slots and Gibbons (1978) demonstrated that the presence of an established dental plaque was necessary for the colonization of B. melaninogenicus. This organism was shown to aggregate with several Gram-positive species, including Actinomyces and Streptococcus, although B. melaninogenicus failed to adhere to oral epithelial cells. A suspension of B. melaninogenicus was introduced into the mouths of volunteers, and subsequently, low numbers were found on epithelial and clean tooth surfaces, but large numbers of this organism were found on pre-existing dental plaque.

Other *in vitro* experiments have indicated a similar series of events occurring with *S. sanguis* and *A. naeslundii* (Ellen and Balcerzak-Raczkowski, 1977). *S. sanguis* was shown to have a high affinity for tooth surfaces, but *A. naeslundii* adhered poorly. When mixed together the two bacteria were seen to aggregate strongly, indicating that pre-formed plaques containing *S. sanguis* would enhance colonization by *A. naeslundii*. It was hypothesized that this sequence of events could account for the delayed establishment of *A. naeslundii* in early plaque deposits *in vivo*.

A similar sequential colonization was demonstrated with extracted teeth *in vitro* with *Capnocytophaga* species and *A. israelii* (Kolenbrander and Celesk, 1983). Amber coloured *Capnocytophaga* plaque formed only on the cementum surface of the teeth, being unable to adhere to the enamel surface. The subsequent inoculation into the system of the *Actinomyces* resulted in patches of white *A. israelii* plaque forming on the *Capnocytophaga* plaque, correlating with the ability of these two organisms to aggregate with each other.

The role of previously established bacteria in the subsequent colonization of another organism was also demonstrated with *A. viscosus* and *Veillonella* species. Pure cultures of *A. viscosus*, but not of *Veillonella*, were able to form plaques on wires *in vitro*. However, in mixed cultures the *Veillonella* were able to form microcolonies within and around the plaque deposits formed by *A. viscosus* (Bladen *et al.*, 1970). A similar association was also noted between *S. mutans* or *S. sanguis* inoculated into gnotobiotic rats with *Veillonella*. Electron microscopy showed colonies of *Veillonella* between masses of streptococci on tooth surfaces (Mikx *et al.*, 1972).

Associations of this kind have also been reported between Veillonella and Actinomyces (Distler and Kroncke, 1981), and Veillonella and Eubacterium saburreum (Mashimo et al., 1981).

1.3.2 Saliva induced homotypic aggregation

Homotypic aggregation occurs where bacteria of a single strain in suspension adhere to each other, either spontaneously or in association with a variety of aggregating factors capable of binding to the bacterial surface. Many oral bacteria are aggregated by suspending them in saliva, a particularly important factor governing oral colonization, although non-oral bacteria generally aggregate poorly in this secretion. Rundegren and Ericson (1981a) tested the ability of bacteria isolated from faeces, skin and the oral cavity to aggregate in saliva. Faecal and skin organisms did not demonstrate appreciable aggregation, whereas the oral strains showed significant aggregation; although the aggregating activities of the individual strains varied considerably.

Saliva is a complex medium and contains more than one aggregating component. For example, Kashket and Donaldson (1972) have shown that different factors in saliva are responsible for the homotypic aggregation of S. sanguis and S. mitior. The two factors varied in their affinity to bind to intact cells and to hydroxyapatite, and in their stability to dialysis. In addition, Rundegren and Ericson (1981b) have shown that the salivary aggregating factors for S. mutans and S. mitior are different, and Kashket and Liberman (1979) and Babu et al. (1986) have described a salivary component specifically involved in the aggregation of S. mutans.

These findings suggest that many different aggregating factors may exist in saliva, each of which, may be capable of interacting with one or more bacterial species (Kashket, Wang and Liberman, 1982).

In addition, it has been shown that the salivary factors responsible for the homotypic aggregation of S. sanguis, S. mitior and S. mutans are different to the factors that mediate adhesion of these streptococci to saliva treated hydroxyapatite (Rosan et al., 1982b). The adsorption of salivary aggregating factors by these bacteria appears to have little effect on the subsequent ability of hydroxyapatite treated with the residual saliva to support adherence of these strains. Conversely, the adsorption of salivary components mediating adherence with hydroxyapatite has little effect on subsequent aggregation. Furthermore, heating saliva at 60°C for 30 minutes significantly reduces bacterial aggregation but has little or no effect on adherence (Rosan et al., 1982b). However, contradicting this report, Ericson and Magnusson (1976) noted that the aggregating factors for S. sanguis, S. mitior and S. mutans showed a distinct affinity for hydroxyapatite, especially the factors that aggregate S. sanguis and S. mutans. Also, Ellen et al. (1983), although using different bacterial genera, concluded the same with A. viscosus and A. naeslundii.

One of the components of saliva responsible for its aggregating activity was isolated from human whole saliva by Hay et al. (1971) using agarose column chromatography. The isolated component was found to consist of 33 per cent protein, 19 per cent carbohydrate, 2.9 per cent N-acetyl neuraminic acid (sialic acid) and substantial amounts of hexosamine. Further tests showed it to be a high molecular weight

glycoprotein existing in solution in a random coil configuration; properties characteristic of salivary mucins (Tabak et al., 1982). It was to found selectively adsorb to hydroxyaptite, and a component with the properties of the above could be isolated from dental plaque. This factor was therefore suggested to play a significant role in the initial selective adhesion of certain oral organisms to the tooth surface, as well as being involved in the aggregation of bacteria in the developing plaque.

Further characterization of salivary aggregating factors using electron microscopy, demonstrated the presence of two types of extracellular structures in salivary aggregates of S. sanguis and S. mitior which were not present with cells incubated without saliva. The S. sanguis aggregating factor consisted of a round globular component and an assymetrical fibrillar unit; the S. mitior aggregating factor only possessed the globular component. The dimensions and shapes of these aggregating factors were consistent with the known high molecular weight of mucinous glycoproteins, and also with the appearance of the intercellular matrix constituents of dental plaque (Kashket, Skobe and Garant, 1978). The suggestion that these aggregating factors were mucins was supported by the isolation of a lectin from the surface of S. sanguis with specificity for salivary mucin (Murray et al., 1982).

Another study investigated the aggregating activity of four isolated salivary glycoproteins (Levine et al., 1978). They found that only two of these glycoproteins demonstrated aggregating activity with S. sanguis and S. mutans, and were shown to be mucin glycoproteins. A notable finding was that the removal of sialic acid

residues from the glycoproteins with neuraminidase resulted in a loss of aggregating activity for S. sanguis, but not for S. mutans; a property confirmed in a later study by Stinson et al. (1982). Also, the loss of the S. sanguis aggregating activity of saliva with neuraminidase or protease treatment correlated with the appearance of free sialic acid (McBride and Gisslow, 1977). Thus, the sialic acid residues of salivary glycoproteins appear to be responsible for the aggregation of certain oral bacteria in saliva.

1.3.3 Crevicular fluid induced homotypic aggregation

A number of strains of S. sanguis were found by Morris and McBride (1983) to aggregate in the presence of non-immune serum and crevicular fluid. All of the strains that aggregated in serum were found to aggregate in saliva, but the converse was not true, ie. some strains that aggregated in saliva did not aggregate in serum. Aggregation was destroyed by treatment of the serum or crevicular fluid with neuraminidase, but adsorption of serum with hydroxyapatite did not reduce its aggregating activity. The aggregation factor was partially purified and found to be a high molecular weight acid glycoprotein, which did not appear to be an immunoglobulin. Therefore, this factor, which has similar characteristics to the salivary S. sanguis aggregating factor, may play a role in the colonization of the gingival crevice region.

Another aggregating component found in both saliva and crevicular fluid was noted recently. The cationic enzyme lysozyme was found to mediate the aggregation of S. sanguis, S. mutans (Golub et al., 1985) and Capnocytophaga gingivalis (Iacono et al., 1985). This

may be associated with the reported lysis of C. gingivalis by lysozyme, and may be significant in providing an antibacterial activity in the gingival crevice (Iacono et al., 1985).

Malamud, Brown and Goldman (1984) suggested that a number of components are present in serum that are potent inhibitors of saliva mediated aggregation of oral streptococci. Several high molecular weight factors were isolated from serum and blood, including serum albumin, fibrinogen, fibronectin and ferritin, which were able to inhibit aggregation at low concentrations. These serum derived factors may also be found in crevicular fluid, a substance similar to serum (Diem and Lentner, 1970; Mason and Chisholm, 1975; Cimasoni, 1983), and so may also influence aggregation in the gingival crevice.

1.3.4 Effect of immunoglobulins on aggregation

Small quantities of serum IgG, IgA and IgM are present in the oral cavity derived from crevicular fluid. However, IgA has been shown to be the major immunoglobulin in the oral cavity and is secreted primarily in saliva (Crawford, Taubman and Smith, 1975). IgA has been shown to comprise approximately 2 per cent of the dry weight of human dental plaque and has been estimated to be 1.6 to 2.7 per cent of the total protein found in plaque (Taubman, 1974).

Liljemark, Bloomquist and Ofstehage (1979) tested the aggregation of 14 strains of S. sanguis, isolated from 5 people, in saliva collected at the same time as the plaque isolates. Where aggregation occurred, it was found that the removal of IgA from the saliva samples caused a decrease in the aggregating activity with 35

per cent of the tests and the stepwise removal of IgA caused a similar stepwise reduction in aggregating activity. Also, the addition of human salivary or colostral IgA to depleted saliva maintained high levels of aggregation.

IgA has also been implicated in the aggregation of other species of oral bacteria. Chromatographical analysis of saliva was shown to yield two peaks with aggregating activity for S. mutans. One peak was a non-specific agglutinin and the other was a specific agglutinin for S. mutans with similar properties to salivary IgA (Everhart, Shreck and Seltizer, 1980). Secretory IgA has also been detected bound to the surfaces of salivary aggregates of Fusobacterium nucleatum (Falkler, Mongiello and Burger, 1979). Thus, IgA appears to be involved in the aggregation of some oral bacteria, and possibly also in the formation of dental plaques.

1.3.5 Heterotypic aggregation

Heterotypic aggregation involves the adherence of cells of two or more bacterial species or strains to each other forming aggregates of mixed cells, and has been shown to occur between many different bacterial species. This type of aggregation has been widely implicated in the colonization of the mouth by exogenous bacter. adhering to pre-formed bacterial accumulations on various oral surfaces and in the formation of dental plaques (McIntire, 1985).

The most extensively studied bacterial species involved in heterotypic aggregation are streptococci and actinomyces. The aggregation of S. sanguis or S. mitior with A. viscosus or

A. naeslundii was shown to be inhibited by amino acid acetylation, protease digestion, the addition of lactose or incubating the streptococci for 30 minutes at 85°C. These observations imply that aggregation is mediated by protein or glycoprotein lectins on the cell surface of one species and by carbohydrate groups on the other (Cisar, Kolenbrander and McIntire, 1979; Kolenbrander and Williams, 1981). McIntire, Crosby and Vatter (1982) investigated the aggregation of A. viscosus and S. sanguis and suggested that the lectin was located on the actinomyces and the carbohydrate group was located on the streptococcus.

Further studies using an aggregation defective mutant of S. sanguis allowed the detection of at least two types of surface components mediating aggregation with A. viscosus and A. naeslundii that were indistinguishable in previous studies (Kolenbrander, 1982). Following this report, Kollenbrander and Williams (1983) tested the aggregating activities of 110 streptococcal isolates from dental plaque against A. viscosus and A. naeslundii. It was found that 61 per cent of the combinations aggregated, and of these 90 per cent were inhibited by lactose. It was concluded that lectin-carbohydrate interactions predominated among the bacterial species which aggregated, but were not the only means by which aggregation occurred.

Mizuno et al. (1983) isolated a highly specific aggregation factor from A. viscosus prepared by lysozyme treatment that could aggregate S. sanguis. On analysis it consisted of 53 per cent cell wall components, 40 per cent polysaccharides and 7 per cent protein. More information on the nature of the cell surface components was obtained using a phage resistant mutant of A. viscosus which had lost

its ability to aggregate with certain oral streptococci. It was suggested that a cell surface structure on A. viscosus may function as a phage receptor and as a binding site for aggregation (Tylenda, Kolenbrander and Delisle, 1983). More recently, a carbohydrate receptor site on the S. sanguis cell surface for A. viscosus was reported to comprise a D-galactose-(1-4)-B-D-glucose sequence, which is related to the inhibition of these species by lactose, galactose and N-acetyl-D-galactosamine (Sato, Koga and Inoue, 1984). These studies indicate that nearly all fresh isolates of these species exhibit specific cell-to-cell interactions (Kolenbrander, Inouye and Holdeman, 1983; Kolenbrander and Celesk, 1983).

Some studies have correlated the aggregating activity of Streptococcus and Actinomyces species with the possession of surface fimbriae or pili. Electron microscopy has shown that the aggregation of A. naeslundii and S. sanguis may be mediated by short tufts of electron dense fuzzy components on the cells surfaces (Ellen and Balcerzak-Raczkowski, 1977). Removal of these fimbriae from A. naeslundii by homogenization has been shown to inhibit homotypic aggregation of this organism (Ellen et al., 1978). Also strains of S. sanguis with peritrichous fimbriae aggregated well with A. viscosus, A. naeslundii and F. nucleatum, whereas strains with tufts of fimbriae could not aggregate with these organisms (Handley et al., 1985). Monoclonal antibodies reacting specifically with A. viscosus fimbriae showed that the lectin activity resides in these structures (Cisar et al., 1980). Thus it appears that heterotypic aggregation between streptococci and actinomyces is mediated predominantly by a lectin in the fimbriae of the actinomyces and a

carbohydrate sequence on the fimbriae of the streptococci.

Heterotypic aggregation, which can be inhibited by lactose, protease or heat, has also been reported between A. viscosus and S. pyogenes, S. agalactiae or Pseudomonas aeruginosa (Komiyama and Gibbons, 1984b). In addition, aggregations thought to be mediated by lectin-carbohydrate interactions and inhibited by lactose were demonstrated between Capnocytophaga ochraceus and A. israelii, A. viscosus, A. naeslundii or S. sanguis (Kolenbrander and Hurst-Calderone, 1981). Thus, lectin-carbohydrate interactions may be found in the aggregation of various bacterial species.

Factors involved in the heterotypic aggregation of other pairs of bacteria have also been partially characterized. Aggregation between Veillonella and S. salivarius was found to be resistant to proteases and unaffected by mono- or di-saccharides, indicating lectin-carbohydrate interactions were not involved (Weerkamp and McBride, 1980a). Also, the aggregation of these organisms did not correlate with the presence of fimbriae (Weerkamp and McBride, 1981), but was found to be mediated by a cell wall associated protein (Weerkamp and Jacobs, 1982). Aggregation between Capnocytophaga species and A. israelii was also found to differ from the lectin-carbohydrate interactions of the streptococci and actinomyces; for example the former were not inhibited by lactose or EDTA (Kagermeier, London and Kolenbrander, 1984; Kagermeier and London, 1986).

McBride and Bourgeau (1975) found that the aggregation of A. viscosus could be mediated by dextran produced by S. sanguis and S. mutans, and with as little as three molecules of dextran per

bacterial cell. This less specific aggregation could also be mediated by dextran isolated from Leuconostoc species (Bourgeau and McBride, 1976). Thus, heterotypic aggregation may be mediated by much less specific factors than lectin-carbohydrate interactions.

1.3.6 Corn cob formations

Heterotypic aggregation of bacteria can occasionally lead to uniquely distinct arrangements of organisms. One such association has been termed the corn cob configuration, which consists of filamentous bacteria covered with coccoid bacteria that vaguely resemble corn cobs. These have been shown by electron microscopy to exist in dental plaque in vivo. Isolation techniques originally identified the two bacteria involved as Bacterionema matruchotii and a S. sanguis like organism (Lancy et al., 1980). It was suggested that the streptococci adhered to the B. matruchotii cell surface via polar fibrillar tufts (Mouton, Reynolds and Genco, 1980).

A more recent study (Lancy et al., 1983) has demonstrated the formation of corn cob structures between F. nucleatum and S. sanguis. Electron microscopy suggested that attachment was due to localized tufts of fimbriae, as in the B. matruchotii system. Further data suggested that several different receptors may be involved. It was proposed that as fusobacteria are among the first anaerobic filaments to colonize subgingival plaque, these interactions could serve as a connecting link between the transformation of supragingival to subgingival plaque.

1.3.7 Effect of pH on aggregation

Some workers have shown that the pH at which aggregation assays are performed can be a critical factor. For example, homotypic aggregation of S. mitior (Abbas and Holme, 1981) and S. sanguis (Kashket and Donaldson, 1972) was unaffected in the pH range of 4.4 to 7.0, but aggregation of S. mitior was decreased at pH 8.0 and completely inhibited at pH 9.0 (Abbas and Holme, 1981). Conversely, pH values below 6.0 induced A. naeslundii to aggregate in culture media or in washed cell suspensions. Similarly, unaggregated suspensions of S. mutans, S. salivarius, S. sanguis and A. viscosus at neutral pH were aggregated when the pH was adjusted to below 6.0 (Miller, Palenik and Stamper, 1978). The pH of saliva is normally in the range of 6.2 to 7.6 (Mason and Chisholm, 1975) which may promote the aggregation of some strains, but would tend to have little effect on most bacteria. However within dental plaque the pH may fall to levels where aggregation could be markedly enhanced.

1.3.8 Effect of bacterial enzymes

Enzymes released by dental plaque bacteria may modify the aggregating activity of potential colonizing microorganisms. Proteases isolated from plaque bacteria by Sato et al. (1983) were shown to inactivate the glucosyltransferase of S. mutans thereby inhibiting the synthesis of glucans involved in aggregation and plaque formation. The enzymes also degraded the cell surface receptors for dextran and glucan on S. mutans, and for salivary agglutinins on S. sanguis, thus inhibiting the homotypic aggregation of these organisms. In addition, heterotypic aggregation of S. sanguis and

A. viscosus was inhibited by the degradation of the A. viscosus cell surface components responsible.

It has also been suggested that the components of saliva conferring aggregating activity may be modified in vivo by plaque associated enzymes. A number of enzymes isolated from Gram-positive plaque bacteria were shown to have a broad spectrum of activity. The hydrolysis of salivary proteins by these enzymes resulted in a loss of aggregating activity (Sato et al., 1983). Clarified saliva has also been shown to contain an endogenous neuraminidase (McBride and Gisslow, 1977) which can inhibit saliva induced homotypic aggregation by the removal of sialic acic residues from the mucin glycoproteins of saliva (Levine et al., 1978).

1.3.9 Aggregation of bacteria implicated in periodontal disease

Many reports have indicated that bacteria must adhere to a surface within the oral cavity to colonize and proliferate, and that bacterial aggregation appears to be an important factor in this process. However, few aggregation studies have been carried out with bacteria thought to be implicated in the aetiology of periodontal disease. Although some of these bacteria have been shown to aggregate homotypically in saliva and heterotypically with other bacterial species, the prevalence of these properties and their importance in colonization are not clear.

1.4 BACTERIAL HYDROPHOBICITY

1.4.1 Hydrophobic interactions and adherence

It has been suggested that the adherence of bacteria to oral surfaces is the result of two essentially different mechanisms, specific and non-specific binding (Gibbons et al., 1985a). Specific binding involves the interaction of complementary sites on the bacterial surface and the colonizable surface, which demonstrate a high affinity. Non-specific binding involves electrostatic or hydrophobic interactions that are of a much lower affinity.

The role of non-specific hydrophobic interactions in bacterial adherence has attracted much interest. Perers et al. (1977) demonstrated that hydrophobic strains of Salmonella typhimurium and Escherichia coli adhered well to the intestinal mucosa of mice, whereas less hydrophobic strains of these organisms showed a decreased ability to adhere to this tissue. More recently Sherman, Houston and Boedeker (1985) found a positive correlation between increased adherence to intestinal epithelial membranes and increased surface hydrophobicity of E. coli.

The ability of strains of another non-oral bacterium, Acinetobacter calcoaceticus, to adhere to human buccal epithelial cells was shown to be related to the surface hydrophobicity of this organism. Bacteria harvested from cultures at different times demonstrated altered hydrophobic properties, which correspondingly altered their adherence. In addition a mutant strain of A. calcoaceticus that adhered poorly was also less hydrophobic than

the wild type (Rosenberg et al., 1981).

Gibbons and Etherden (1983) studied the hydrophobic activity of a number of oral bacteria: including strains of A. viscosus, A. naeslundii, S. sanguis, S. mitior and B. gingivalis which were highly hydrophobic; also strains of B. intermedius, B. melaninogenicus, S. salivarius and S. mutans which were less hydrophobic; and strains of H. actinomycetemcomitans which were hydrophilic. The adherence of these bacteria to saliva treated hydroxyapatite was then assessed and found to generally correlate with their hydrophobic properties; the more hydrophobic strains showing a higher affinity.

Studies using mutants of S. sanguis have also revealed a link between adherence and hydrophobicity. Gibbons et al. (1983b) isolated a non-hydrophobic mutant of S. sanguis that adhered poorly to saliva treated hydroxyapatite compared to its wild-type. Fives-Taylor and Thompson (1985) used a mutagen to select seventeen mutants of S. sanguis that were deficient in adherence properties and were subsequently found to be less hydrophobic.

Also, Gibbons, Etherden and Moreno (1983a) investigated the relationship between hydrophobic interactions and low and high affinity binding sites for S. sanguis on saliva treated hydroxyapatite. The high affinity sites involved interactions between specific neuraminidase-sensitive receptors that became saturated at high bacterial concentrations. The low affinity sites predominating at high bacterial concentrations were inhibited by sodium thiocyanate, a chaotropic agent which interferes with hydrophobic bonding. Thus it

was suggested that at high bacterial concentrations, hydrophobic interactions predominate in adhesion. Ciardi et al. (1983) measured the hydrophobic potentials of oral streptococci and saliva treated and untreated hydrophobic polystyrene and less hydrophobic glass. They concluded that the order and strength of adsorption of oral streptococci to the test surfaces was influenced by the hydrophobic and electrostatic potentials of the bacterial surfaces and the saliva formed pellicles.

The importance of hydrophobic interactions in vivo was suggested by Svanberg, Westergren and Olsson (1984). They obtained two hydrophobic and two hydrophilic streptomycin resistant strains of S. mutans and introduced them into the mouths of volunteers. The hydrophobic strains were found to colonize better than the hydrophilic strains. In addition, two studies (Weiss et al., 1982; Rosenberg, Judes and Weiss, 1983a) reported that the majority of bacteria in dental plaque were hydrophobic. Also, emulsan, an amphipathic polysaccharide which inhibits hydrophobic interactions, was shown to desorb over 70 per cent of the normal flora adherent to buccal epithelial cells (Rosenberg, Gottlieb and Rosenberg, 1983).

Conditions within the oral cavity may influence bacterial hydrophobicity. For example, Rogers, Pilowsky and Zilm (1984) reported that S. mutans and S. milleri cells were more hydrophobic when grown slowly in continuous culture with a mean generation time of seventeen hours, compared with bacteria grown with a one hour mean generation time. Bacteria in the oral cavity have been reported to grow slowly (Gibbons, 1984) and this may therefore increase their surface hydrophobicity in vivo and influence their colonization.

It is clear that there are many reports expounding the involvement of bacterial surface hydrophobicity in adherence reactions. However, a number of reports contradict these findings. Rosenberg, Rottem and Rosenberg (1982) reported that a strain of non-oral Proteus mirabilis was hydrophilic yet adhered well to buccal epithelial cells, and further, that mutant strains with different degrees of increased hydrophobicity adhered poorly. Lambden et al. (1979) obtained mutants of Neisseria gonorrhoeae with increased adhesion to buccal epithelial cells. None of the mutants showed increased hydrophobicity, indicating that hydrophobic interactions were not involved in adherence.

The hydrophobic surface properties of S. mutans, S. sanguis and S. salivarius were determined by Olsson and Westergren (1982). The results indicated that the S. salivarius strains were more hydrophobic than the S. sanguis and S. mutans strains and, although S. salivarius adheres comparatively well to buccal epithelial cells, it adheres poorly to saliva treated hydroxyapatite. It is clear that hydrophobic interactions cannot account for such specificity. Robinson and Handley (1984) divided strains of S. sanguis into three groups according to their distibution of fimbriae. The different groups adhered in varying proportions to saliva treated hydroxyapatite, but no correlations were evident with respect to hydrophobicity and adherence between the three groups. Hence, it may be theorized that the association between adherence and hydrophobicity is the result of some bacterial cell surface components which confere adherence, also being hydrophobic in nature. However, this does not necessarily mean that the hydrophobic properties of these components are responsible

for adherence. Thus, the link between adherence and hydrophobicity may only be circumstantial.

1.4.2 Hydrophobic bacterial surface structures

Various bacterial surface structures have been associated with hydrophobic surface properties. The distribution of different types of fimbriae on strains of S. sanguis was found to be related to hydrophobicity (Robinson and Handley, 1984). Gibbons et al. (1983b) isolated a non-hydrophobic mutant of S. sanguis which was defective in the synthesis of fimbriae, and Fives-Taylor and Thompson (1985) isolated seventeen non-hydrophobic mutants of S. sanguis that had also lost their fimbriae. Thus, fimbriae may be responsible for the surface hydrophobicity of these and other bacteria.

Different amino acids demonstrate varying degrees of hydrophobicity, so the composition of amino acids incorporated into cell surface structures may influence the hydrophobic properties of the cells. Amino acid analysis of cell walls of S. sanguis revealed the presence of several amino acids with hydrophobic side chains and it was suggested that these amino acids were responsible for the hydrophobic properties of this organism (Nesbitt, Doyle and Taylor, 1982a). The presence of cell surface proteins was also suggested to confer hydrophobic properties to S. pyogenes since non-hydrophobic mutants were shown to have lost these proteins (Wadstrom et al., 1984). Similarly, a high molecular weight protein that could be isolated from the cell walls of hydrophobic S. mutans could be isolated only from culture supernatants with hydrophilic variants, and it was concluded that hydrophilic mutants could produce this protein

but could not incorporate it into their cell walls (McBride et al., 1984). Therefore, there is evidence to suggest that cell surface proteins may confer hydrophobic properties on certain bacteria.

Courtney et al. (1985) showed that the hydrophobic properties of S. pyogenes were inhibited by treatment with fibronectin. Also, pretreatment of fibronectin with lipotechoic acid (LTA) was shown to prevent this hydrophobicity-inhibiting-property of fibronectin. It was therefore concluded that LTA is responsible for the hydrophobicity of S. pyogenes. Also, Miorner et al. (1983) reported that the hydrophobicity of strains of S. pyogenes correlated with their LTA surface content, and suggested that LTA is the major factor determining the surface hydrophobicity of S. pyogenes. However, although LTA appears to confer hydrophobic properties on S. pyogenes, McBride et al. (1984) reported that the LTA content of non-hydrophobic S. mutans mutants was the same as hydrophobic wild-type strains.

1.4.3 Factors affecting bacterial hydrophobicity

Exogenous substances binding to bacterial cell surfaces may also influence hydrophobicity. Human serum albumin, fibrinogen and immunoglobulin G adsorbed to the surfaces of streptococci were found to alter their hydrophobic potentials (Miorner et al., 1980; Courtney et al., 1985). Likewise, salivary components adsorbed to streptococci isolated from the oral cavities of monkeys were reported to cause either an increase or a decrease in surface hydrophobicity depending on the bacterial strain tested (Beighton, 1984). This effect was investigated by Babu, Beachey and Simpson (1986) who isolated a high molecular weight component of human saliva that bound to S. sanguis

and caused a decrease in hydrophobicity. Thus, the adsorption of components from saliva or crevicular fluid by bacteria in the oral cavity may affect their hydrophobic properties.

The conditions under which bacteria are grown *in vitro* have been shown by a number of reports to affect hydrophobicity. Beighton (1984) noted that streptococci grown in Todd-Hewitt broth were more hydrophobic than those grown in brain-heart-infusion-yeast-extract broth. Olsson and Westergren (1982) reported that *S. mutans* grown in three different nutrient-rich media gave similar results, but when grown in a defined medium the different strains gave variable results. Rogers *et al.* (1984) found that *S. mutans* and *S. milleri* were more hydrophobic when grown in media containing glucose compared with fructose. It therefore appears that the nutrients available to an organism will influence its hydrophobicity, presumably by affecting the synthesis of cell surface components.

Several reports have noted differences in the hydrophobic activity of bacteria harvested from cultures at different phases of growth. *S. pyogenes* (Ofek, Whitnack and Beachey, 1983), *A. calcoaceticus* (Rosenberg *et al.*, 1981) and *Serratia marcescens* (Kjelleberg, Lagercrantz and Larsson, 1980) have all been shown to be more hydrophobic when in the late log phase or stationary phase of growth. However, *Proteus mirabilis* strains were shown to be unaffected by the age of the culture (Rosenberg *et al.*, 1982), as were *S. mutans* strains grown on blood agar plates (Westergren and Olsson, 1983). Similarly, Olsson and Westergren (1982) observed that *S. mutans* grown in batch culture was unaffected by age. However, when grown in a chemostat at constant pH late stationary phase cells were

less hydrophobic. This result was explained by the adsorption of different medium components at different pH levels.

It is evident that a number of environmental factors affect bacterial hydrophobicity *in vitro*. Such factors will presumably have comparable effects *in vivo*. It follows, therefore, that the hydrophobicity of bacteria will be influenced by the local environment of their surrounding habitat. This may vary considerably in different sites such as the gingival crevice and the tooth surface, especially with respect to pH and nutrient availability. Therefore, the hydrophobic activity of bacteria *in vivo* may depend on their intra-oral location.

1.4.4 Role of hydrophobicity in colonization

Opinion as to the actual importance of hydrophobic interactions in bacterial adherence to oral surfaces is divided. Rosan, Eifert and Golub (1985) suggested that research into bacterial hydrophobicity should be suspended in favour of the study of the specific molecular interactions involved in adhesion. Rosenberg *et al.* (1983b) concluded that hydrophobic interactions are primarily responsible for mediating the adherence of most oral bacteria to the various surfaces within the oral cavity. Further work therefore seems necessary to resolve this controversy.

Correlations between adherence and hydrophobicity have been made considering usually just one type of surface, ie. buccal cells or hydroxyapatite. The specific nature of adherence regarding different types of surface has largely been disregarded. Also, both adherence

and hydrophobicity *in vitro* have been shown to be affected by experimental variables such as buffer composition (Yamazaki, Ebisu and Okada, 1981; Eifert *et al.*, 1984; Rogers *et al.*, 1984). Yet few studies have standardized these factors for both adherence and hydrophobicity assays, which could make subsequent correlations questionable. These factors may well contribute to the controversy surrounding the significance of bacterial surface hydrophobicity in adherence and aggregation.

1.5 Bacteria associated with periodontal disease

1.5.1 Aetiology of periodontal disease

The microorganisms in the oral cavity present an unusual situation within the human body in that the normal bacterial flora is often associated with localized disease processes, namely caries and periodontal disease. Periodontal disease is a general term used for several conditions in which the supporting tissues of the teeth are attacked (Patters, 1983) and is a major cause of tooth loss (Kay and Blinkhorn, 1986). However, it is clear from the literature cited in the preceding sections that although there is considerable knowledge concerning factors involved in the oral colonization of streptococci and actinomyces, there is relatively little information concerning the Gram-negative bacteria associated with periodontal disease. Since one of the main aims of this thesis was to study such bacteria, the following section reviews briefly the evidence implicating particular bacterial species in the aetiology of periodontal disease.

There is good evidence indicating that bacteria play a major role in the aetiology of periodontal disease. Epidemiological studies have shown a strong positive correlation between the amount of dental plaque and the severity of chronic gingivitis (Ash, Gitlin and Smith, 1964; Socransky, 1970; Listgarten, 1976; van Palenstein Helderman, 1981a). In support of this theory it was demonstrated that withdrawl of all measures of oral hygiene in healthy persons with clinically healthy gingiva resulted in dental plaque formation and development of gingivitis within 10 to 21 days. Furthermore, resumption of plaque removal quickly restored gingival health (Loe, Theilade and Jensen,

1965; Holm-Pedersen, Agerbaek and Theilade, 1975). Thus it is reasonable to assume that an increase in plaque mass exerts a greater pathogenic effect on the periodontal tissues.

However, the mere presence of a pathogen does not necessarily indicate that periodontal destruction is taking place. The effect of additional factors must be considered, for example, the numbers of bacteria present, their spacial location, the local environmental conditions, the influence of other organisms present, and the susceptibility of the host tissues (Socransky, 1977)

1.5.2 Development of plaque and periodontal disease

Various investigators have studied the qualitative development of plaque and have shown that the composition of supragingival and subgingival plaque differs in patients with periodontal disease as compared to patients with a healthy periodontium. Unfortunately, the proportions of different species of bacteria isolated in various studies tend to be inconsistent due to the use of different isolation and identification techniques and to innate variations between the microflora of different subjects and even of different sites within the same individual (Evian, Rosenberg and Listgarten, 1982; Moore *et al.*, 1984).

Cultural studies of the gingival crevice area at healthy sites have shown that supragingival and subgingival plaque is similar (van Palenstein Helderman, 1981b), with a predominance of streptococci, mainly *S. sanguis* and *S. mitior*. *Actinomyces* species are also frequently present in both sites, sometimes in relatively high

numbers, and a wide range of other microorganisms can be found, but mostly in low numbers (Ritz, 1967; Slots, 1977b; Loesche and Syed, 1978; Zambon et al., 1981; Theilade, Theilade and Mikkelsen, 1982). Slots (1977b) also reported that streptococci were the most common subgingival isolates (39 per cent), mainly S. sanguis and S. mitior, along with Actinomyces species (32 per cent), mainly A. israelii (26 per cent). Low proportions of Gram-negative rods were present (13 per cent), consisting of mainly Bacteroides and Fusobacterium species. Other organisms present included species of Veillonella (2 per cent), Peptostreptococcus (0.8 per cent) and Neisseria.

Supragingival and subgingival plaque at chronic gingivitis sites is generally more abundant than that associated with healthy sites (Listgarten, 1976). The subgingival microflora associated with gingivitis, compared to normal sites, generally possesses fewer streptococci and increased proportions of Gram-negative rods and cocci, actinomyces, fusiforms, spirilla and spirochetes (Holm-Pedersen et al., 1975; van Palenstein Helderman, 1975; Socransky et al., 1977; Loesche and Syed, 1978; White and Mayrand, 1981; Moore et al., 1982a; Savitt and Socransky, 1984). Slots et al. (1978) recorded similar findings including a marked increase in the proportions of black-pigmented Bacteroides, particularly B. intermedius, and increased proportions of Capnocytophaga and Peptostreptococcus species. Although the proportions of bacterial species isolated in healthy and chronic gingivitis subjects were different, the same species could be found in both conditions.

Chronic periodontitis is usually associated with larger accumulations of supragingival and subgingival plaque than is normally found with chronic gingivitis (Listgarten, 1976). The decrease in numbers of streptococci and increase in numbers of Gram-negative rods noted with chronic gingivitis is generally more pronounced with chronic periodontitis. Notable species of Gram-negative rods that have been found in elevated proportions include F. nucleatum, B. gingivalis, B. intermedius and H. actinomycetemcomitans (Dwyer and Socransky, 1968; Newman et al., 1976; Tanner et al., 1979; Spiegal et al., 1979; Zambon et al., 1981; Mashimo et al., 1983; Tanner, Socransky and Goodson, 1984; Slots et al., 1986). However, Williams, Pantalone and Sherris (1976) isolated increased proportions of Actinomyces species and similar levels of Gram-negative rods from periodontitis sites compared to healthy sites. Slots (1977a) found that of the bacteria isolated from subgingival plaque at periodontitis sites, 90 per cent were anaerobes and 75 per cent were Gram-negative. Black-pigmented Bacteroides constituted 32 per cent of the total isolates, with B. gingivalis predominating and B. intermedius also forming a large proportion. Actinomyces species were present in lower proportions (16 per cent) than in healthy sites, with A. israelii the most common species (10 per cent). Streptococci formed only 6 per cent of the total isolates. In addition, as with chronic gingivitis, all of the species associated with chronic periodontitis can also be isolated from healthy sites, although less frequently and in lower proportions.

The subgingival microflora of juvenile periodontitis and of adult chronic periodontitis were found to be similar, with high levels

of Gram-negative anaerobic rods (60 per cent). A large proportion of these organisms found in juvenile periodontitis appeared to be Capnocytophaga species and B. oralis. The remaining organisms were assumed to be strains of B. corrodens, B. melaninogenicus and F. nucleatum, although many of the Gram-negative isolates could not be placed into known genera. Gram-positive rods constituted 15 per cent of the total flora, streptococci 6 per cent and Peptostreptococcus species 6 per cent (Slots, 1976; Newman et al., 1976; Savitt and Socransky, 1984). Similarly, Moore et al. (1985) listed B. gingivalis, B. intermedius, Peptostreptococcus species and A. israelii among isolates found in increased proportions, but noted no difference with the Capnocytophaga species. Slots, Reynolds and Genco (1980) implicated a bacterial species in the aetiology of periodontal disease that had not been widely recognized, namely Haemophilus actinomycetemcomitans. This organism was found in 90 per cent of diseased sites in juveniles, but in only 20 per cent of normal sites in juveniles. The association of H. actinomycetemcomitans with juvenile periodontitis was doubted by Asikainen et al. (1986), but was confirmed by the majority of reports, including Mandell and Socransky (1981), Slots and Rosling (1983), Zambon, Christersson and Slots (1983), Mashimo et al. (1983), Mandell (1984), Savitt and Socransky (1984), Moore et al. (1985), Zambon (1985) and Tempro, Bochacki and Zambon (1986). The latter report noted that H. actinomycetemcomitans, B. intermedius and B. gingivalis were recovered alone or in various combinations in 89 per cent of young adults with advanced periodontitis and in 100 per cent of juvenile periodontitis patients. These reports therefore strongly implicate H. actinomycetemcomitans in juvenile periodontitis, along with several other species.

More than 250 bacterial species may be found associated with periodontal disease (Slots, 1982b). Consequently, it has been difficult to define which species play a major role in the aetiology of periodontal disease. The large number of recent studies on the cultivable flora associated with periodontal disease have produced a confusing and often contradictory array of likely periodontopathic bacteria and no definitive proof yet exists for the involvement of particular species. However, the frequency with which several species have been isolated in different studies in increased numbers from diseased sites compared to healthy sites, suggests that certain species are potential aetiological agents in periodontal disease. These species include: Bacteroides species, particularly B. gingivalis and B. intermedius; F. nucleatum; H. actinomycetemcomitans; Capnocytophaga species; and Peptostreptococcus species.

1.6 Aims of this study

Although a number of bacterial species have been associated with periodontal disease, little is known about the factors and mechanisms involved in the colonization of the mouth by these microorganisms. Therefore, the main aim of this thesis was to investigate the early stages in the colonization of the mouth by bacteria implicated in periodontal disease with respect to bacterial adherence, aggregation and hydrophobicity. The plan of investigation was as follows.

Bacteria were selected for study to include species believed to be involved in the pathogenesis of periodontal disease, namely, Bacteroides gingivalis, Bacteroides intermedius, Capnocytophaga species, Haemophilus actinomycetemcomitans and Peptostreptococcus species, as well as species representative of the normal commensal flora, namely, Veillonella species, Actinomyces israelii, Streptococcus salivarius and Streptococcus sanguis. A freshly isolated strain and a type culture were selected for each species studied giving a total of eighteen test bacteria.

The ability of the test bacteria to adhere to various oral surfaces was investigated. Since many of the bacterial strains selected for study were difficult to visualize microscopically using conventional assay methods, new techniques were developed which were used initially to study adherence to exfoliated human buccal epithelial cells (Chapter 2). However, since buccal cells present several problems when studying the effect of various factors on adherence (eg. contamination by commensal bacteria or varied exposure to saliva), a new method was developed using HeLa cell monolayers

which could be pretreated with saliva or serum to mimic buccal or crevicular epithelial cells, respectively (Chapter 3). Finally adherence of the test bacteria to saliva treated tooth enamel was investigated using a novel technique (Chapter 4).

A spectrophotometric method was used to test homotypic aggregation of the test bacteria in: (i) clarified, mixed saliva, (ii) a buffer with ionic constituents similar to saliva, and (iii) buffered physiological saline. From these results a suitable suspending medium was chosen to investigate heterotypic aggregation of the test bacteria (Chapter 5). Due to the volume and complexity of the data obtained, a computer programme was developed to aid the analysis of the results.

It has been hypothesized that the hydrophobic properties of bacterial cell surfaces influence bacterial adherence and aggregation, although conflicting reports are presented in the literature. Therefore, the hydrophobic properties of the test bacteria were determined, in the same buffers used to assay adherence and aggregation, to determine if any correlations could be found between these phenomena (Chapter 6).

The information compiled in this study was summarized in the final chapter for each species tested to assess which of the factors studied may be the most important in colonization of the oral cavity by different bacteria (Chapter 7). Ultimately, this study aimed to identify the primary colonization mechanisms of these bacteria, which may help in the development of new methods for the prevention and treatment of periodontal disease.

CHAPTER 2

AN *IN VITRO* METHOD TO STUDY THE ADHERENCE OF BACTERIA TO BUCCAL EPITHELIAL CELLS

2.1 INTRODUCTION

The literature cited in Section 1.2.1 strongly suggests that the ability to adhere to a variety of surfaces is important in the colonization of the oral cavity. Adherence enables bacteria to survive on surfaces exposed to the washing action of saliva that would otherwise remove them. A widely employed technique for assessing adherence to oral surfaces was first described by Gibbons and van Houte (1971) who used exfoliated buccal epithelial cells as the test surface. Most studies on the adherence of oral bacteria to buccal cells have used streptococci; for reviews see van Houte (1983) and Gibbons (1984). Very few, notably Slots and Gibbons (1978) and Okuda *et al.* (1981), have studied the adherence of anaerobic Gram-negative oral bacteria to buccal cells. These organisms are more difficult to study than Gram-positive streptococci for a number of reasons: they are more difficult to isolate, are more fastidious when grown in culture, are smaller in size, and stain and contrast with buccal cells poorly using conventional bacterial stains such as Gram's. Therefore, the use of existing buccal cell assay methods for anaerobic Gram-negative bacteria are not ideal.

These problems have resulted in a lack of standardized information on the ability of oral Gram-negative bacilli to adhere to

epithelial surfaces. Consequently, relatively little is known about the factors involved in the colonization of the human mouth by these bacteria, many of which have been implicated in the aetiology of periodontal disease. This study was undertaken in an attempt to obtain more information on these factors using assay methods developed to exclude some of the sources of error inherent in existing techniques.

2.2 MATERIALS AND METHODS

2.2.1 Bacteria studied

The bacteria used in this study consisted of ten different species of oral bacteria, including Gram-negative and Gram-positive rods and cocci. The bacteria selected were chosen to represent bacterial species believed to be involved in the pathogenesis of periodontal diseases, as well as species regarded as common members of the normal commensal microbial flora not involved in periodontal disease. The bacteria used are listed below with a brief description of the characteristics of each species.

Bacteroides gingivalis

The name Bacteroides melaninogenicus was originally used to describe a group of Gram-negative, anaerobic, rod-shaped bacteria that produce brown or black pigmented colonies when grown on blood agar. This species was eventually divided into three subspecies, namely subspecies melaninogenicus, intermedius and asaccharolyticus (Holdeman and Moore, 1973). These subspecies were subsequently re-classified as distinct species (Finegold and Barnes, 1977). It was further proposed that B. asaccharolyticus be divided into two separate species; B. asaccharolyticus (non-oral isolates) and B. gingivalis (oral isolates) (Coykendall, Kaczmarek and Slots, 1980). The heterogeneity of these two species is now well established (Slots and Genco, 1979; Mouton et al., 1981; Slots, 1981; van Steenbergen, Vlaanderen and de Graaf, 1981).

The primary habitat of B. gingivalis is the gingival crevice where it has been widely implicated in the aetiology of chronic periodontitis and also to some extent in gingivitis (Slots, 1979; White and Mayrand, 1981; Moore et al., 1983 and 1985; Takazoe, Nakamura and Okuda, 1984; Slots and Genco, 1984; Haffajee et al., 1986; Slots et al., 1986). B. gingivalis was selected for study because of this association with periodontal disease.

Bacteroides intermedius

B. intermedius, a black pigment-producing, anaerobic, Gram-negative rod, was previously known as B. melaninogenicus subspecies intermedius (Holdeman and Moore, 1973), but has now been designated as a distinct species (Johnson and Holdeman, 1983).

B. intermedius is a common inhabitant of the gingival crevice and has been widely implicated in periodontal disease. B. intermedius appears to be involved in experimental gingivitis, pregnancy gingivitis, acute necrotizing ulcerative gingivitis and juvenile and adult periodontitis (Zambon et al., 1981; Loesche et al., 1982; Slots, 1982b; Moore et al., 1982b, 1983 and 1985; Haffajee et al., 1986; Slots et al., 1986). B. intermedius was also chosen for study as a species typically implicated in periodontal disease.

Capnocytophaga species

Capnocytophaga species are facultatively anaerobic, Gram-negative, fusiform rods which require carbon dioxide (Socransky et al., 1979). The Capnocytophaga species were previously known as

Bacteroides ochraceus (Holdeman and Moore, 1973) before being reasigned to a separate genus consisting of three species, namely C. ochraceus, C. sputigena and C. gingivalis (Leadbetter et al., 1979; Socransky et al., 1979). However, the criteria used in speciation are not totally conclusive, for example, of the 68 strains tested by Socransky et al. (1979), 10 could not be speciated. Therefore, the isolates used in this study were designated only as Capnocytophaga species.

Capnocytophaga species have been found in increased proportions in gingivitis (van Palenstein Helderman, 1975; Slots et al., 1978; Savitt and Socransky, 1984) and juvenile periodontitis (Slots, 1976; Newman et al., 1976; Newman and Socransky, 1977; Mashimo et al., 1983; Savitt and Socransky, 1984). They have also been shown to cause massive loss of alveolar bone in monoinfected gnotobiotic rats with minimum plaque formation (Irving et al., 1976; Crawford et al., 1977) as seen in juvenile periodontitis (Patters, 1983). However, Slots and Rosling (1983) and Moore et al. (1985) found the proportion of Capnocytophaga species in juvenile periodontitis to be similar to those in healthy gingival sulci. The role of Capnocytophaga species in adult chronic periodontitis is also not clear and recently Socransky et al. (1986) listed Capnocytophaga species among the bacteria that may be beneficial to the host in relation to adult periodontitis. Although the association of Capnocytophaga species with periodontal disease is uncertain, the evidence suggesting an aetiological role was considered sufficient to include it amongst the bacteria chosen for study.

Haemophilus actinomycetemcomitans

Isolates with the characteristics of the Gram-negative, facultatively anaerobic rods, H. actinomycetemcomitans, were first described in 1912 and were named Bacterium actinomycetem comitans. This was changed to Actinobacillus actinomycetemcomitans in 1929 (Phillips, 1973) and then to Haemophilus actinomycetemcomitans by Potts, Zambon and Genco in 1985 in view of the organisms genetic and serological relationship to the genus Haemophilus.

H. actinomycetemcomitans has been widely implicated in the aetiology of juvenile periodontitis, but it is not clear if this organism is involved in adult periodontitis (Slots et al., 1980; Mandell and Socransky, 1981; Mashimo et al., 1983; Slots and Rosling, 1983; Page et al., 1983; Mandell, 1984; Savitt and Socransky, 1984; Moore et al., 1985; Zambon, 1985; Mandell et al., 1986; Slots et al., 1986; Tempro et al., 1986). This species was included on the basis of its association with juvenile periodontitis and its possible association with adult periodontitis.

Haemophilus aphrophilus

The strain of H. aphrophilus used in this study was originally identified as H. actinomycetemcomitans and was selected to pair with the type strain of this species. However, in the latter stages of this study, the fresh isolate was found to possess characteristics more similar to H. aphrophilus. This strain was isolated and identified according to the method of Slots (1982a) whereby isolated colonies of H. actinomycetemcomitans were said to be catalase-positive

and to demonstrate characteristic star-like inner structures on a selective medium, which distinguished this species from H. aphrophilus and a few other possible contaminating organisms. However, Tanner et al. (1982) noted that some strains of H. actinomycetemcomitans were catalase-negative and some H. aprophilus strains were catalase-positive. In addition, a number of colonies with star-like inner structures have been isolated in Glasgow Dental Hospital with biochemical profiles pertaining to H. aphrophilus. For this reason the biochemical profiles of the fresh and type haemophili were compared with newly established criteria, based mainly on the production of acid from lactose and sucrose also reported by Kilian and Schiott (1975) and by Tanner et al. (1982). The results confirmed the identity of the type strain as H. actinomycetemcomitans, but the fresh strain appeared to more closely resemble H. aphrophilus. Therefore, the strains selected for study included a fresh isolate of H. aphrophilus and a type strain of H. actinomycetemcomitans.

H. aphrophilus is a Gram-negative, facultatively anaerobic rod, originally described by Khairat in 1940 and reported to be closely related to H. actinomycetemcomitans (Tanner et al., 1982; Coykendall, Setterfield and Slots, 1983; Potts and Berry, 1983; Potts et al., 1985). H. aphrophilus has been found in association with various extra-oral infections, although the role of this organism in periodontal diseases is not clear. However, H. aphrophilus is indigenous to dental plaque (Kilian and Schiott, 1975; Kilian, Prachyabrued and Theilade, 1976) and has been found in larger proportions in subgingival plaque compared with supragingival plaque from patients with chronic periodontitis (Moore et al., 1983;

Liljemark et al., 1984) and has been positively correlated with gingivitis (Moore et al., 1982a).

Peptostreptococcus species

The Peptostreptococcus species are Gram-positive, anaerobic cocci found commonly in dental plaque and in clinical infections (Gibbons et al., 1964b; Holdeman, Cato and Moore, 1977). There are five species of Peptostreptococcus (Rogosa, 1973b), however the methods in general use for the differentiation of the various species are impractical and largely inconclusive (Smith, Ross and Cumming, 1984). Peptostreptococcus and Peptococcus are the only genera of anaerobic Gram-positive cocci normally found in the oral cavity (Smith, 1982) and may be differentiated by their sensitivity to novobiocin (Wren, Eldon and Dakin, 1977). The isolates used in this study were determined to be Peptostreptococcus species using this method, but were not speciated.

Although Peptostreptococcus species have not been as widely implicated in periodontal disease as have certain Gram-negative rods, increased numbers of Peptostreptococcus species have been associated with gingivitis (Slots et al., 1978; Moore et al., 1982a), adult periodontitis (Moore et al., 1983) and juvenile periodontitis (Moore et al., 1982b). In addition, Haffajee et al. (1986) included Peptostreptococcus micros among the bacteria likely to be responsible for destructive periodontal disease. The pathogenic potential of this genus is clearly demonstrated by its frequent involvement in clinical infections (Pien, Thompson and Martin, 1972) and from pathogenicity testing in animals (Brook and Walker, 1984), although comparatively

little work has been done with the Peptostreptococcus species. The evidence implicating the Peptostreptococcus species in periodontal disease was considered sufficient to warrant their inclusion in this study.

Veillonella species

The Veillonella are small, Gram-negative, anaerobic cocci, which, in general, do not ferment carbohydrates (Rogosa, 1973a). The genus has been divided into seven species which are exceedingly similar phenotypically. Three of these species have been isolated from humans, namely V. parvula, V. dispar and V. atypica (Mays et al., 1982). Because of the phenotypic similarities of the Veillonella, differentiation to species level was not attempted and the strains used were designated as Veillonella species.

Veillonella constitute one of the most common genera found in the oral cavity, mainly in plaque and on the tongue dorsum (Gibbons and van Houte, 1975). Veillonella species are not considered to be pathogenic (Smith, 1982) nor have they been associated with the aetiology of periodontal disease, although Sveen and Skaug (1980) found that lipopolysaccharide from Veillonella stimulated bone resorption. Recently, Socransky et al. (1986) listed Veillonella species among the possibly beneficial bacteria in relation to periodontal disease. Veillonella were included for study because of the common occurence of this commensal with which bacteria implicated in periodontal disease might interact.

Actinomyces israelii

The Actinomyces are Gram-positive, non-spore forming, facultatively anaerobic filaments, divided into six species (Slack, 1973; Holdeman et al. 1977). Five of these species are found in the oral cavity, mainly in dental plaque but also on the tongue dorsum and in saliva (Gibbons and van Houte, 1975; Holdeman et al., 1977).

A. israelii is frequently found associated with human infections, but its role in periodontal disease is uncertain (Jordan, 1982). For instance, A. israelii was shown to form plaques and to initiate caries and periodontal disease in gnotobiotic rats (Jordan, 1982). Also, human subjects with chronic periodontal disease were found to have significantly increased levels of serum antibodies to A. israelii compared to non-diseased control subjects (Nisengard and Beutner, 1970; Gilmour and Nisengard, 1974). Thus A. israelii appears to be able to colonize the gingival crevice area and to initiate host responses thought to be linked to periodontal disease. However, Crawford et al. (1977) found that A. israelii inoculated into gnotobiotic rats did not cause periodontal disease, and Socransky et al. (1986) included A. israelii among the possibly beneficial species in relation to periodontal disease. In addition, A. israelii is frequently found in large proportions in healthy gingival crevice areas (Slots, 1977b) and although some reports note increases in Actinomyces species in gingivitis (Loesche and Syed, 1978) and in periodontitis (Williams et al., 1976), these reports are more the exception than the rule. Thus, it is unlikely that this species is significant in the aetiology of periodontal disease. The inclusion of A. israelii in this study was as a normal commensal found on a variety

of oral surfaces and in the gingival crevice where it would be ideally placed to interact with bacteria implicated in periodontal disease.

Streptococcus salivarius

S. salivarius is a facultatively anaerobic, Gram-positive coccus (Deibel and Seeley, 1973) found in high proportions on the tongue dorsum, on the buccal mucosa and in saliva, but in low proportions in dental plaque (Gibbons and van Houte, 1975). Therefore, as might be expected considering this organisms preferred niche, S. salivarius has not been associated with periodontal disease. S. salivarius was included for study as a normal commensal with which bacteria implicated in periodontal disease might interact, and also for comparative purposes because of the wide use of this organism in other studies.

Streptococcus sanguis type I

S. sanguis is a facultatively anaerobic, Gram-positive coccus (Deibel and Seeley, 1973) found in high proportions in dental plaque and also in saliva and on mucosal surfaces (Gibbons and van Houte, 1975). S. sanguis is found in high numbers in the healthy gingival crevice region and its proportions decrease as plaque matures and periodontal disease progresses (Newman et al., 1976; Williams et al., 1976; Slots, 1977a and b; Loesche and Syed, 1978; Slots et al., 1978; Moore et al., 1985; Socransky et al., 1986). It was also reported that S. sanguis did not induce periodontal disease when inoculated into gnotobiotic rats (Crawford et al., 1977). S. sanguis was included for study because it has a wide distribution within the oral

cavity, and for comparative purposes because of the large volume of information in the literature concerning the adherence, aggregation and hydrophobicity of this organism. Both isolates used in this study were type I strains.

Both fresh and type strains of bacteria have certain advantages and disadvantages associated with their use in adherence assays. Type cultures have the advantage of being well characterized and readily available to all workers. However, it has been reported that as a result of repeated subculture bacteria can lose certain adherence confering components (Williams and Gibbons, 1975; Orstavik and Orstavik, 1982; Westergren and Olsson, 1983). Therefore, for each of the species selected, with the exception of the haemophili, a type strain was obtained in addition to a freshly isolated oral strain, giving a total of 18 test bacteria as listed below:-

Bacteroides gingivalis P4

Bacteroides gingivalis W83

Bacteroides intermedius P2

Bacteroides intermedius NCTC 9336

Capnocytophaga species P2

Capnocytophaga species ATCC 27872

Haemophilus aphrophilus P5

Haemophilus actinomycetemcomitans NCTC 9710

Peptostreptococcus species P2

Peptostreptococcus species NCTC 9807

Veillonella species P3

Veillonella species NCTC 11463

Actinomyces israelii P2

Actinomyces israelii NCTC 10215

Streptococcus salivarius P2

Streptococcus salivarius NCTC 8618

Streptococcus sanguis P1

Streptococcus sanguis NCTC 7863

2.2.2 Source of type cultures

The type cultures were obtained from the National Collection of Type Cultures (NCTC) (Central Public Health Laboratory, London, England) with two exceptions: Capnocytophaga species ATCC 27872 was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A), and B. gingivalis W83 was kindly provided by Dr. J. M. Hardie, London Hospital Medical College, London, England.

2.2.3 Isolation of fresh strains

Freshly isolated strains were obtained from patients suffering from chronic periodontitis who were attending Glasgow Dental Hospital. All nine isolates came from five patients coded P1 to P5; five isolates from patient P2 and one isolate from each of the other four patients.

After removal of supragingival plaque using a periodontal currette, subgingival plaque was collected from the periodontal pocket with a fresh currette. Plaque samples were placed in 0.1 ml of Anaerobe Blood Broth (ABB) (Gibco Europe Ltd, Paisley, Scotland) in sterile plastic bijou bottles. The plaque samples were dispersed by

passing 10 times through a 25 gauge, 0.5 x 25 mm needle (Beckton, Dickinson U.K. Ltd., Oxford, England). The plaque suspension was then diluted with ABB from neat to 10^{-4} and 0.1 ml volumes of appropriate dilutions were plated onto the following five culture media; 5 per cent horse blood agar, campylobacter agar (for Bacteroides and Capnocytophaga species), mitis-salivarius agar (for Streptococus species), teepol agar (for Veillonella species) and tryptic soy-serum-bacitracin-vancomycin (TSBV) agar (for Haemophilus species). Details of the suppliers and constituents of these media are listed in Appendices 1 to 5.

The plates were incubated at 37°C in an atmosphere of 85 per cent nitrogen, 10 per cent hydrogen and 5 per cent carbon dioxide in a Forma model 1024 anaerobe chamber (Forma Scientific, Marietta, Ohio, U.S.A.) except for the mitis-salivarius and TSBV agar plates which were incubated in air with 5 per cent carbon dioxide in a Qualitemp 80 MI CO_2 incubator (LTE, Oldham, England) for between 24 and 72 hours. Different colony types were subsequently plated out for purity onto 5 per cent horse blood agar plates.

2.2.4 Identification of study strains

Both fresh isolates and type cultures were identified using a combination of Gram-staining characteristics, atmospheric requirements, ability to grow on selective media, colonial morphology and biochemical profiles. Streptococcus species were identified using the API 20 Strep system (API Systems S.A., Montalieu Vercieu, France) which consists of 20 biochemical tests performed in 20 pre-prepared plastic microtubes. The biochemical profiles of the

anaerobic bacteria and the facultative Gram-negative bacteria were determined using the Minitek miniaturised microorganism differentiation system (BBL Microbiology Systems, Cockeysville, M.D., U.S.A.) which consists of 15 biochemical tests.

In addition to the above tests, a fluorescent antibody technique was used to confirm the identity of the fresh A. israelii isolate (CDC, Atlanta, Georgia, U.S.A.). Also, the sensitivities of the anaerobic streptococci to 5 μg novobiocin discs were determined to differentiate between peptococci and peptostreptococci, according to the method of Wren et al. (1977). Both strains were sensitive, indicating they were of the latter genus.

2.2.5 Maintenance of cultures

In order to preserve the freshly isolated bacteria, freeze dried stock cultures were prepared within three subcultures of isolation. This aimed to minimise the possiblity of phenotypic variation as a result of continuous subculturing on laboratory media. Freeze dried stock cultures of the type strains were also prepared. Ampoules were opened after the strains had been subcultured on average five times.

Cultures were also maintained by subculturing on 5 per cent horse blood agar plates. The anaerobic bacteria were grown initially at 37°C for 24 - 48 hours in a Forma anaerobe chamber, then placed in an area in the anaerobe chamber at room temperature. The facultative bacteria were grown at 37°C in air with 5 per cent carbon dioxide in a Qualitemp 80 MI CO_2 incubator for 24 - 48 hours, then stored in air at 4°C.

2.2.6 Preparation of broth cultures

Broth cultures were prepared by inoculating 20 ml volumes of anaerobic blood broth supplemented (ABB) (Appendix 6) (Gibco Europe, Glasgow, Scotland) in glass McCartney bottles from fresh plate cultures. These were incubated at 37°C in an atmosphere of 85 per cent nitrogen, 10 per cent hydrogen and 5 per cent carbon dioxide in a Forma anaerobe chamber for 24 to 120 hours. Cultures were grown until the stationary phase of growth, determined from growth curves of the respective bacteria. For most of the test bacteria, the time required was 24 hours, but the B. gingivalis, Capnocytophaga and Veillonella strains required 48 hours and the A. israelii strains required 120 hours. Broths used to grow Veillonella species were supplemented with 1 ml of a 20 per cent, filter sterilized, sodium lactate solution (Sigma Chemical Co., Poole, Dorset, England) giving a final broth concentration of 1 per cent.

One exception to the above was A. israelii P2 which formed large floccular masses when grown in ABB, which proved to be unsuitable for experimental purposes. A. israelii P2 was, therefore, grown in tryptic soy broth (TSB) (Appendix 7) (Gibco Europe Ltd.) which produced only small floccular masses similar to those produced by A. israelii NCTC 10215 grown in ABB. The small aggregates of both A. israelii strains were removed by centrifugation at 100 g for 2 minutes followed by filtration through a 12 μm pore size 25 mm diameter polycarbonate filter (Nucleopore Corp., Pleasanton, C.A., U.S.A.). This procedure rendered uniform A. israelii suspensions of predominantly single cells which did not spontaneously aggregate.

2.2.7 Preparation of bacterial suspensions

Stationary phase cultures were decanted into sterile disposable plastic universals (Nunc Inter Med, Kamstrup, Denmark) and centrifuged at 3000 g for 10 minutes in an MSE super minor centrifuge (MSE Scientific Instruments, Crawley, England). The resultant pellet was resuspended in 20 ml of saliva ions buffer (SIB) (Appendix 8) and centrifuged again, finally resuspending the bacteria in SIB to a concentration of 10^8 cells per ml according to optical density measurements, as described below.

2.2.8 Determination of bacterial concentrations

The optical density measurements of the bacterial suspensions at a concentration of 10^8 cells per ml were determined separately for each of the 18 bacteria under study. For each strain a series of dilutions of bacterial suspensions was prepared in SIB. The optical density of each of the dilutions was then determined at 520 nm in a Pye Unicam SP 8-100 spectrophotometer (Pye Unicam, Cambridge, England) using plastic disposable cuvettes with a path length of 10 mm (Sarstedt Ltd., Leicester, England).

The bacterial concentration of one of the dilutions was then determined as follows. A suspension was diluted further by a factor of 10^{-3} and 1 ml of this dilution was added to 1 ml of 0.025 per cent acridine orange (Hopkin and Williams Ltd., Chadwell Heath, Essex, England) in distilled water, previously filtered through a 0.45 μm pore size Sterifil D-HA filtration unit (Nihon Millipore, Kogyo K.K., Yonezawa, Japan). After two minutes, the stained suspension was

filtered through a 0.6 μm pore size, 25 mm diameter polycarbonate filter (Nucleopore Corporation, Pleasanton, CA,. U.S.A.) mounted on a DEFT (direct epifluorescent filter technique) manifold (Micromeasurements Ltd., Saffron Walden, Essex, England).

The DEFT manifold (Figure 2.1) consists of a stainless steel housing on which up to five filters can be mounted in filter units consisting of a sintered glass base beneath a filter tower, between which the filters are sandwiched. The weight of the filter tower forms a seal against the filter and a negative pressure is applied to the manifold from a Venturi pump drawing the suspension in the filter tower through the filter. The stained bacteria are thus deposited evenly over the exposed area of the filter, determined by the internal diameter of the filter tower. To prevent the possibility of the bacterial suspension leaking between the filter and the filter tower housing, the filter was sealed against the base of the filter tower with a small amount of silicone grease (BDH Chemicals Ltd., Poole, England).

The filters, with attached bacteria, were then removed from the filter units, air dried and mounted on 1.0 - 1.2 mm thick, 26 x 76 mm glass microscope slides with Uvinert immersion oil (BDH Chemicals Ltd., Poole, England) under 25 x 25 mm glass coverslips. The mounted filters were examined under ultra violet light at a magnification of X 1000 using a Nikon Optiphot microscope (Nippon Kogaku K.K., Tokyo, Japan). Fifty fields were randomly selected and the numbers of bacteria on the filter in each field were counted. This procedure was performed a minimum of three times for each bacterium studied and the mean value was calculated.

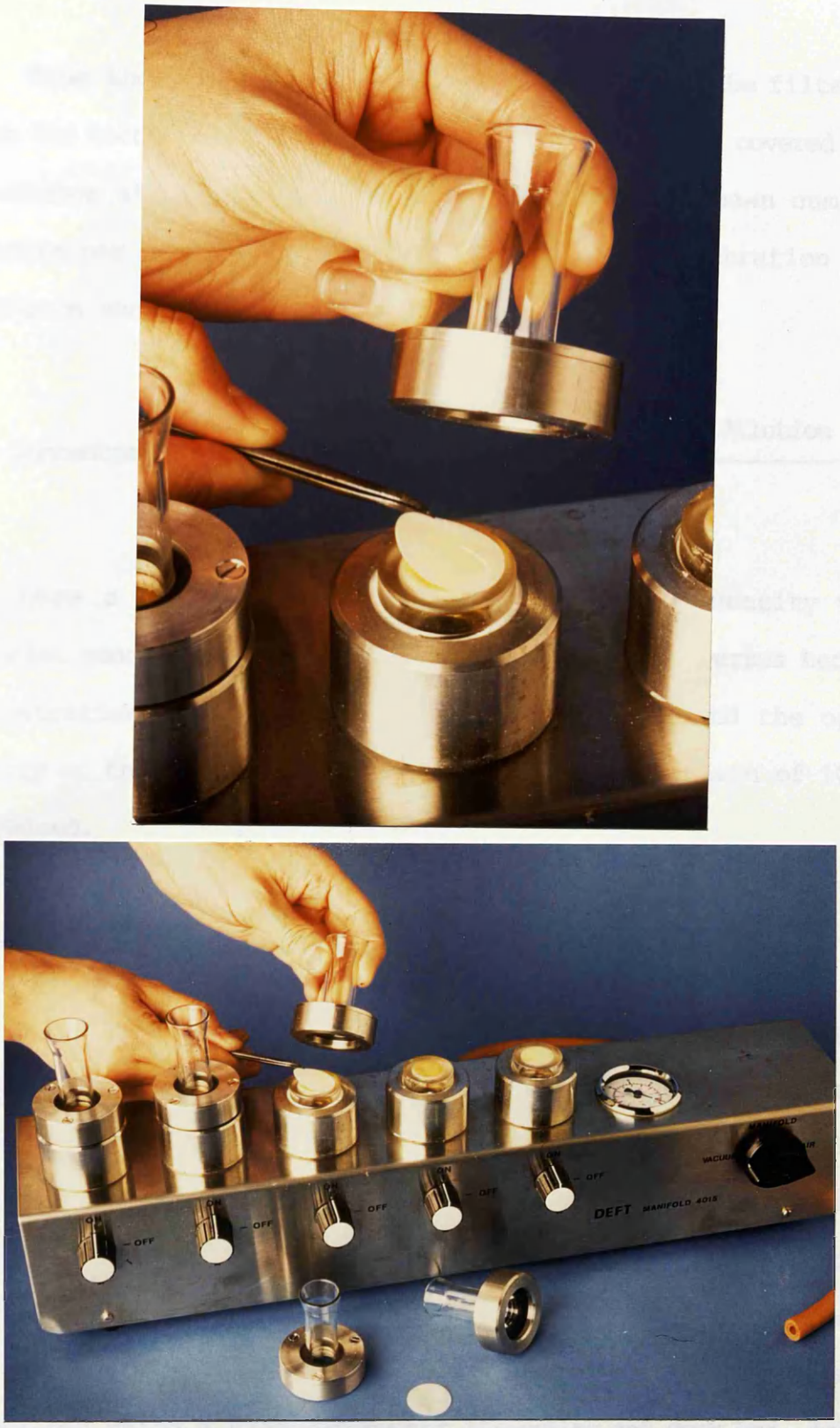


Figure 2.1 DEFT manifold filter unit showing the placement of a filter on a sintered glass base beneath a filter tower.

From the known dilution factors, the area of the filter over which the bacteria are deposited, the area of the field covered by the microscope at a magnification of X 1000 and the mean number of bacteria per microscope field, the bacterial concentration of the suspension was calculated from the following equation:

$$\text{Concentration} = \frac{\text{number bacteria x filter area x dilution}}{\text{microscope field area}}$$

This gave a series of pairs of data of optical density versus bacterial concentration. A graph of optical density versus bacterial concentration was then constructed (Figure 2.2) and the optical density of the bacterial suspension at a concentration of 10^8 was determined.

2.2.9 Preparation of buccal cell suspensions

Exfoliated buccal epithelial cells were collected by gently scraping the inside of the cheeks of a healthy 25 year old dentate donor with a wooden tongue depressor and suspending the dislodged cells in 5 ml of SIB. The cells were then washed twice by centrifuging at 2000 g for 5 minutes and resuspending in 5 ml of SIB. Finally, the buccal cells were centrifuged as above and resuspended in 1 ml of SIB. The cell concentration of this suspension was then determined using a haemocytometer (Hawksley and Sons Ltd., Lancing, England) and the final volume was adjusted to give a final buccal cell concentration of 10^5 per ml.

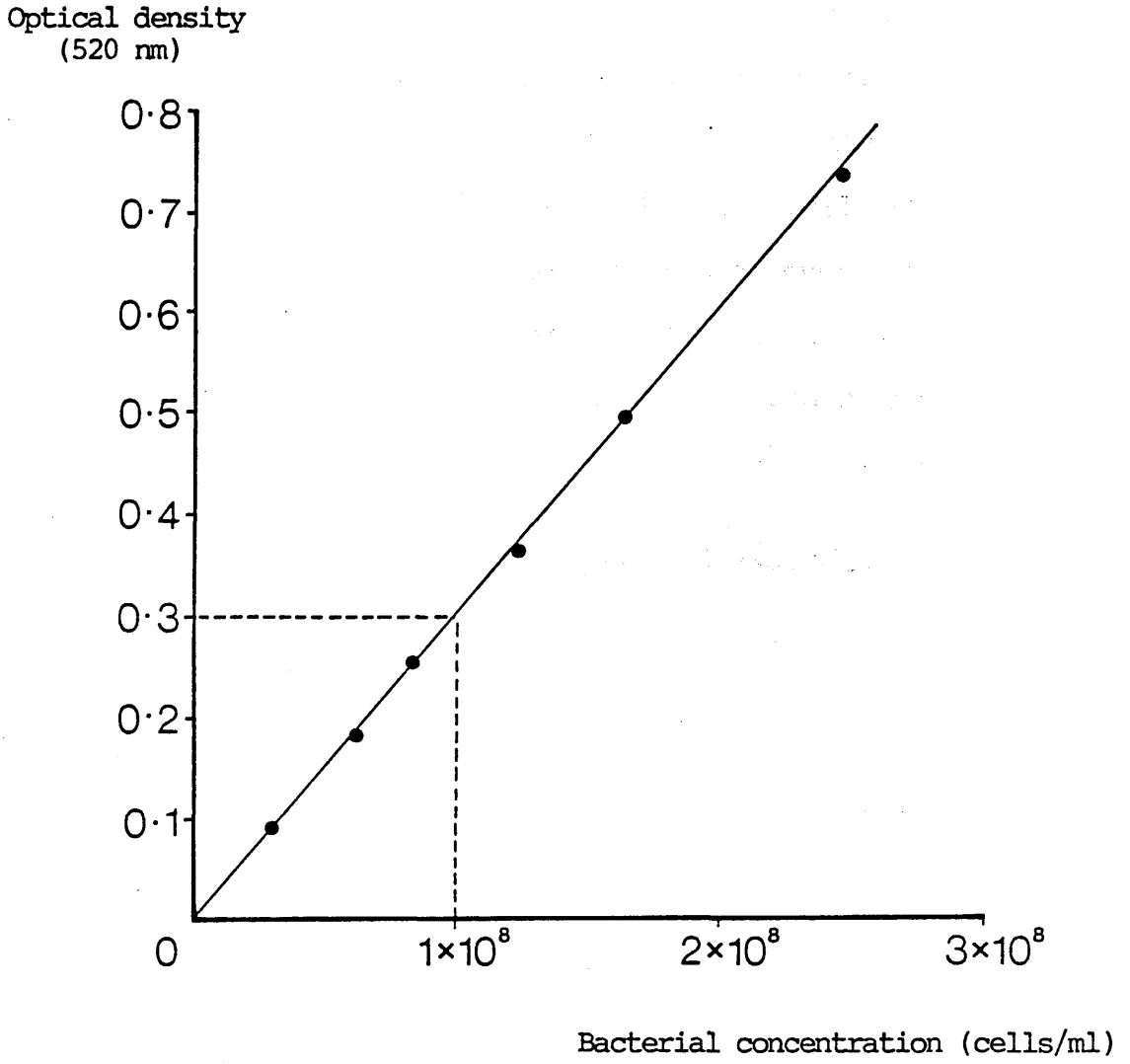


Figure 2.2 An example of a typical plot used to determine the optical density of a bacterial suspension at a concentration of 10^8 bacteria per ml.

In the example shown, 10^8 cells/ml = O.D. of 0.3 at a wavelength of 520 nm (data obtained for S. sanguis P1).

2.2.10 Buccal cell adherence assay

The adherence assay was carried out in plastic disposable bijou bottles (Sterilin Ltd., Feltham, England) and consisted of 0.1 ml aliquots of buccal cell suspension with 0.1 ml of bacterial suspension in SIB. Controls consisted of 0.1 ml of buccal cells with 0.1 ml of SIB, and were used to determine background counts of indigenous bacteria. Test and control samples were then incubated at 37°C on an orbital shaker (A. Gallenkamp and Co. Ltd., London, England) at a speed of 60 rpm for 60 minutes.

Following incubation, the contents of each bijou were diluted with 5 ml of SIB to minimise any further attachment. The buccal cells were then collected on 25 mm diameter polycarbonate filters with a pore size of 12 μm (Nucleopore Corp., Pleasanton, CA., U.S.A.) mounted on a DEFT manifold (Figure 2.1, Section 2.2.8). A negative pressure of 5 mm Hg was applied to the manifold and the filters with retained buccal cells were washed with 2.5 ml of SIB, three times, to remove unattached bacteria. The pore size of 12 μm was small enough to retain the buccal cells without gross distortion but large enough to allow unattached bacteria to be washed through the filter unit, leaving only buccal cells with adherent bacteria on the polycarbonate filter.

2.2.11 Staining procedure

The buccal cells with attached bacteria were stained on the filter while within the filter manifold using 2.5 ml of a 0.025 per cent solution of acridine orange (Hopkin and Williams Ltd., Chadwell

Heath, Essex, U.K.) in pH 3.3, 0.1 M citrate/sodium hydroxide buffer, for 2 minutes. The filter was then washed twice with two further rinses of SIB (2.5 ml). Excess background fluorescence was quenched with 2.5 ml of a 0.01 per cent solution of potassium permanganate in distilled water for 2 minutes, and the cells were given a further two rinses with SIB (2.5 ml).

Before use the acridine orange and potassium permanganate solutions were filtered through 0.45 μm pore size Sterifil D-HA filtration units (Nihon Millipore, Kogyo K.K., Yonezawa, Japan) to ensure that no particulate matter was present, which could produce microscopic artifacts.

2.2.12 Bacterial enumeration

The stained filters were removed from the filter units, air dried and mounted on 1.0 - 1.2 mm thick, 26 x 76 mm glass microscope slides with non-fluorescent Uvinert immersion oil (BDH Chemicals Ltd., Poole, England) under 25 x 25 mm glass coverslips. The mounted filters were then examined under ultra violet light at a magnification of X 1000 using a Nikon optiphot microscope (Nippon Kogaku K.K., Tokyo, Japan). Buccal cells were selected at random and the number of bacteria adherent to each cell was quantified visually.

In order to standardise the results as far as possible, buccal cells were only included for counting in the study if they were:

(i) Morphologically normal cells, possesing a single nucleus and with a round entire edge.

(ii) Single cells, spatially separated from any other cell.

(iii) Cells that were flat, not folded.

(iv) Cells free from obvious non-bacterial debris that could interfere with counting.

The mean number of indigenous bacteria adherent to the washed buccal cells usually amounted to less than two bacteria per buccal cell. Very occasionally the indigenous bacterial count was found, on counting at the end of the assay procedure, to excede this value, in which case the entire assay was discarded and repeated on another occasion.

The minimum number of buccal cells to be counted was determined using cumulative mean plots (Chalkey, 1943). This method involves counting the number of adherent bacteria to a large number of buccal cells, and calculating the progressive mean number adhering for an increasing number of fields. The data is then plotted as the mean number of adhering bacteria versus the number of buccal cells counted. The mean values show a large variance with a small number of fields, but the variance decreases as the number of buccal cells counted increases. The number counted that gives a variance within 10 per cent of the final mean is usually considered acceptable for biological purposes (Weibel, 1969) and is taken as the minimal sample size. The number of cells required to be counted, varied, depending on the mean number of bacteria adhering, with larger counts giving a smaller variance. Therefore, to ensure uniformity of the results, fifty buccal cells were counted on each occasion an assay was performed.

2.2.13 Statistical analyses

The assay procedure was repeated a minimum of three times on different days for each bacterium studied. The mean numbers of bacteria attached per buccal cell, determined for each repeat assay, were averaged and the standard errors of the means and coefficients of variation were calculated using standard statistical formulae.

Since the data obtained was shown to be non-parametric by graphical means, the significance of differences between results were determined using the Mann-Whitney U test.

2.3 RESULTS

2.3.1 Determination of bacterial concentrations

Optical densities of the bacterial suspensions at a concentration of 10^8 bacteria per ml were found to range from 0.032 to 0.450 (Table 2.1). This relatively wide range was probably due to variations in the relative size of different bacteria. For example, the Streptococcus species were much larger than the Bacteroides species when viewed microscopically and correspondingly gave much larger optical densities at the same cell concentrations. Also, the fresh Capnocytophaga isolate appeared microscopically very similar to the type strain, except that the cells were approximately three times longer, and consequently gave an optical density three times greater with the same number of cells per ml.

Bacteria deposited on the polycarbonate filter stained a bright orange or apple green when viewed under ultra-violet light. The proportions of orange to green bacteria varied from strain to strain, but had no effect on counting. The deposited bacteria contrasted well against the non-staining filter and made counting easy.

The stained bacteria were deposited evenly over the polycarbonate filter surface, except for a few patchy areas devoid of bacteria. This is due to the presence of non-wettable areas in the filter. Consequently, a larger number of fields needed to be counted to ensure that the variance of the mean was within ten per cent. It is of interest to note that the manufacturers now claim to have produced improved completely wettable filters.

Table 2.1 Optical densities required to give suspensions of 10^8 bacteria per ml at a wavelength of 520 nm.

Bacterium	Optical density
B. gingivalis P4	0.032
B. gingivalis W83	0.081
B. intermedius P2	0.120
B. intermedius NCTC 9336	0.049
Capnocytophaga species P2	0.355
Capnocytophaga species ATCC 27872	0.101
H. aphrophilus P5	0.067
H. actinomycetemcomitans NCTC 9710	0.070
Peptostreptococcus species P2	0.190
Peptostreptococcus species NCTC 9807	0.210
Veillonella species P3	0.079
Veillonella species NCTC 11463	0.227
A. israelii P2	0.268
A. israelii NCTC 10215	0.403
S. salivarius P2	0.450
S. salivarius NCTC 8618	0.290
S. sanguis P1	0.303
S. sanguis NCTC 7863	0.193

2.3.2 Buccal cell adherence results

All of the bacteria under study were readily visualised adhering to buccal cells and could be counted accurately. The bacteria stained either a bright orange or apple green, contrasting with the lighter staining, pale green buccal cells (Figures 2.3 and 2.4). The nuclei of the buccal cells stained a brighter green, but sufficient contrast with the bacteria was still maintained. The proportion of orange to green bacteria varied from strain to strain, but this produced no difficulty in counting adherent bacteria.

Examples of the raw data obtained are presented for H. actinomycetemcomitans NCTC 9710 in Tables 2.2 a, b and c and for Peptostreptococcus species NCTC 9807 in Tables 2.3 a, b and c, which represent three repeat assays for each bacterium tested, with 50 buccal cells counted for each assay. The number of adherent bacteria on each buccal cell was recorded, along with the corresponding control buccal cells.

The numbers of bacteria adhering to the individual buccal cells varied between 0 and 31 with H. actinomycetemcomitans NCTC 9710 (Table 2.2) and between 0 and 147 with Peptostreptococcus species NCTC 9807 (Table 2.3). Frequency distributions of the results from Tables 2.2 and 2.3 are shown in Figures 2.5 and 2.6, respectively. The histograms indicate that the results are not normally distributed, therefore non-parametric statistical analyses were used. Although the results of only two strains are presented in Tables 2.2 and 2.3, they are representative of the results obtained with the remaining 16 test bacteria.

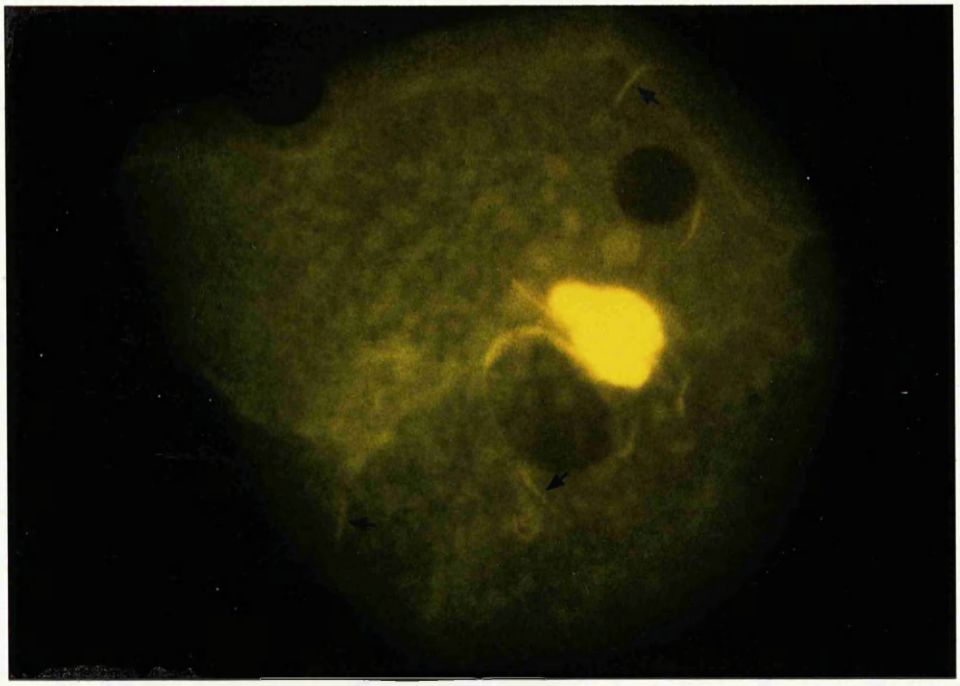


Figure 2.3 Capnocytophaga species P2 (3 arrowed) adhering to a buccal cell. Magnification X 1000.

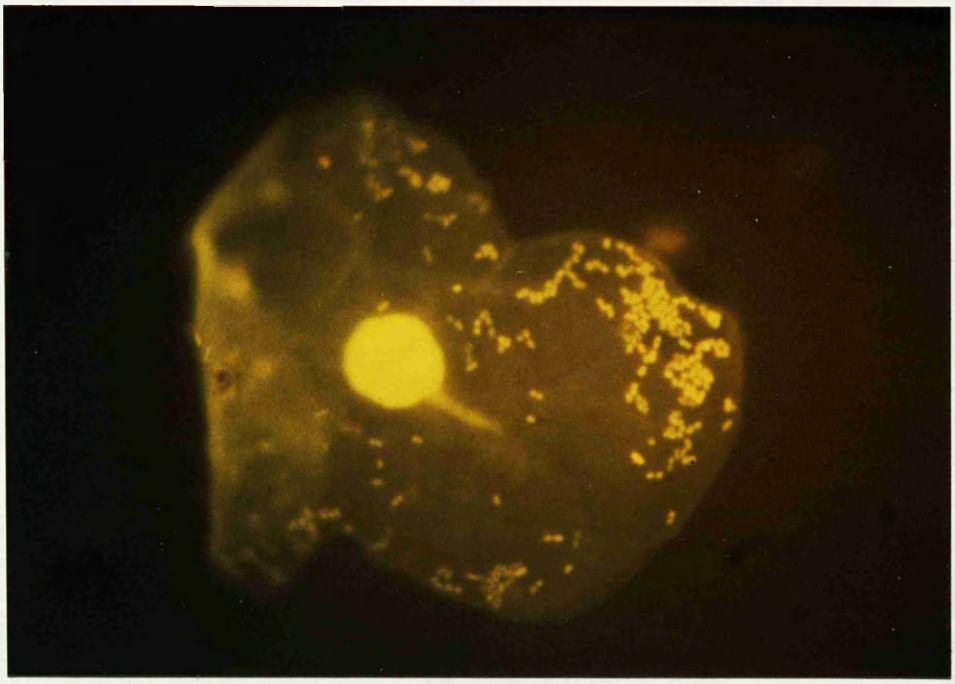


Figure 2.4 S. sanguis P1 adhering to a buccal cell. Magnification X 1000.

Table 2.2(a) An example of the raw data obtained with Haemophilus actinomycetemcomitans NCTC 9710.

Experiment 1.

Buccal cell number		Number of bacteria per buccal cell			
		test		control	
1	26	8	31	0	0
2	27	13	5	0	0
3	28	0	6	0	0
4	29	7	2	0	1
5	30	11	5	0	0
6	31	4	25	0	0
7	32	0	7	0	0
8	33	9	29	0	0
9	34	4	8	0	0
10	35	3	9	0	0
11	36	2	3	0	0
12	37	2	13	0	0
13	38	0	10	0	0
14	39	0	10	0	0
15	40	3	2	0	0
16	41	0	3	0	0
17	42	4	0	0	0
18	43	0	5	0	0
19	44	2	3	0	0
20	45	10	5	0	0
21	46	21	8	0	0
22	47	31	5	0	2
23	48	5	11	0	0
24	49	8	1	0	0
25	50	7	7	0	0
Total		367		3	
SEM		1.09		0.04	
Mean		7.34		0.06	
Test - control		7.3		0	

Table 2.2(b) An example of the raw data obtained with Haemophilus actinomycetemcomitans NCTC 9710. Experiment 2.

Buccal cell number		Number of bacteria per buccal cell			
		test		control	
1	26	15	8	0	0
2	27	6	7	0	0
3	28	2	1	0	1
4	29	7	4	0	0
5	30	16	10	0	0
6	31	7	5	0	0
7	32	2	6	0	0
8	33	5	4	0	0
9	34	1	7	0	0
10	35	0	7	1	0
11	36	11	8	0	0
12	37	1	5	1	0
13	38	3	3	0	0
14	39	18	1	0	0
15	40	8	4	0	0
16	41	10	9	1	0
17	42	19	25	0	0
18	43	0	11	0	0
19	44	15	4	0	0
20	45	4	6	0	0
21	46	3	4	0	0
22	47	0	2	0	0
23	48	12	0	0	0
24	49	4	0	0	0
25	50	17	11	0	0
Total		338		4	
SEM		0.80		0.04	
Mean		6.76		0.08	
Test - control		6.7		0	

Table 2.2(c) An example of the raw data obtained with Haemophilus actinomycetemcomitans NCTC 9710. Experiment 3.

Buccal cell number		Number of bacteria per buccal cell			
		test		control	
1	26	0	6	0	0
2	27	12	8	0	0
3	28	18	7	0	0
4	29	6	5	0	2
5	30	3	0	0	0
6	31	7	13	0	0
7	32	14	4	0	0
8	33	2	12	1	0
9	34	7	8	0	0
10	35	6	6	0	0
11	36	6	13	1	0
12	37	1	7	0	0
13	38	22	8	0	0
14	39	5	30	0	0
15	40	12	5	0	0
16	41	8	14	0	0
17	42	2	8	0	0
18	43	6	7	1	0
19	44	8	8	0	0
20	45	9	4	0	0
21	46	12	11	0	0
22	47	13	9	0	0
23	48	23	1	0	0
24	49	8	5	0	0
25	50	2	26	0	0
Total		437		5	
SEM		0.90		0.05	
Mean		8.74		0.10	
Test - control		8.6		0	

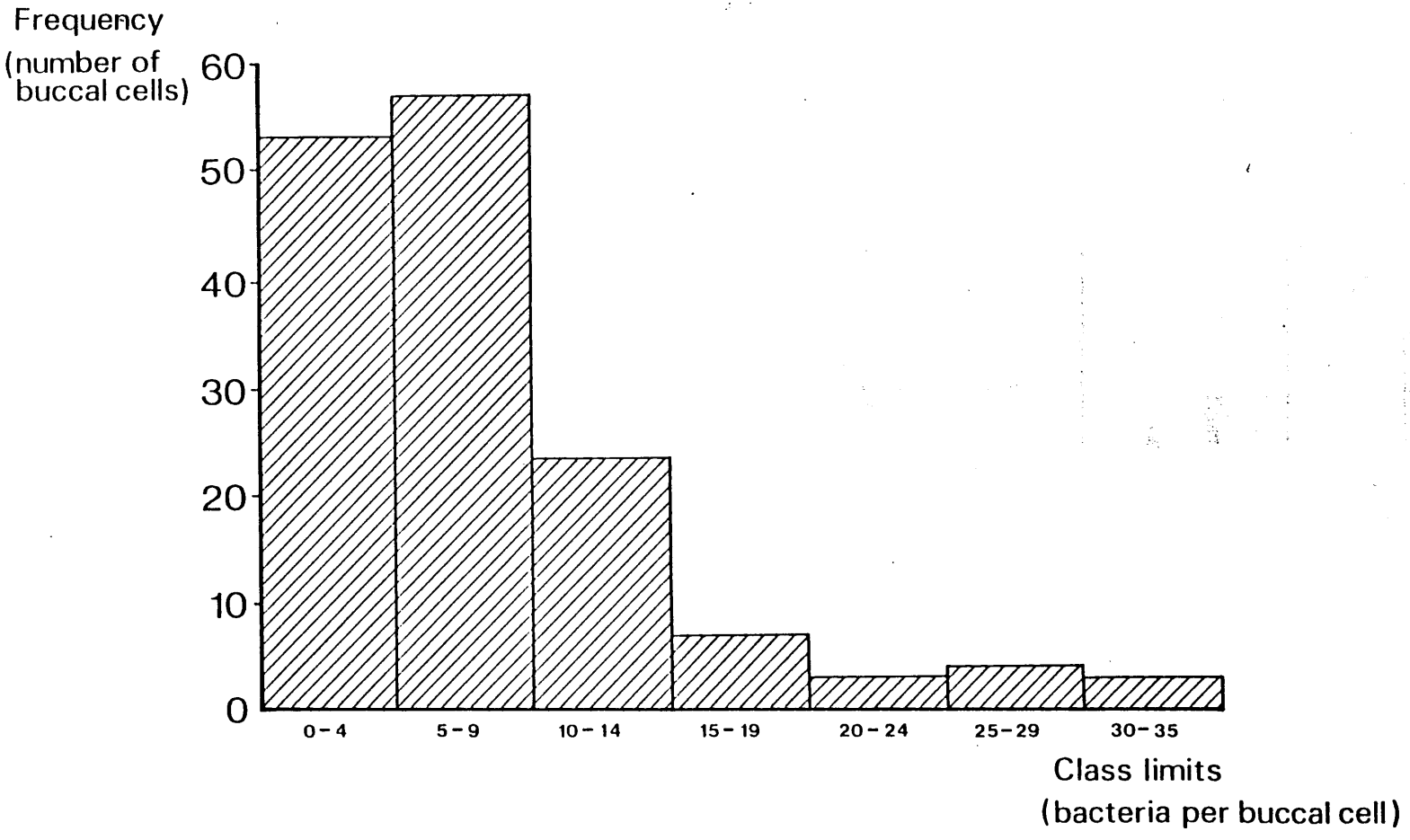


Figure 2.5 Frequency distribution of the numbers of H. actinomycetemcomitans NCTC 9710 adhering per buccal cell.

Table 2.3(a) An example of the raw data obtained with Peptostreptococcus species NCTC 9807.

Experiment 1.

Buccal cell number		Number of bacteria per buccal cell			
		test		control	
1	26	14	15	0	0
2	27	61	41	0	0
3	28	30	63	0	1
4	29	26	27	0	0
5	30	28	18	0	0
6	31	137	27	0	0
7	32	32	27	0	0
8	33	19	89	0	0
9	34	27	48	0	0
10	35	19	138	0	0
11	36	62	55	0	0
12	37	45	2	0	0
13	38	29	44	0	0
14	39	12	21	0	0
15	40	37	41	0	5
16	41	53	15	0	0
17	42	46	60	10	0
18	43	21	75	0	2
19	44	73	49	0	0
20	45	88	31	0	0
21	46	75	39	0	0
22	47	7	8	0	0
23	48	26	36	0	0
24	49	44	23	0	0
25	50	63	23	0	0
Total		2089		18	
SEM		4.07		0.22	
Mean		41.8		0.36	
Test - control		41		0	

Table 2.3(b) An example of the raw data obtained with Peptostreptococcus species NCTC 9807.

Experiment 2.

Buccal cell number		Number of bacteria per buccal cell			
		test		control	
1	26	63	94	0	1
2	27	73	28	0	0
3	28	92	34	0	0
4	29	46	44	0	0
5	30	70	78	0	0
6	31	82	23	0	0
7	32	70	91	0	0
8	33	40	49	0	0
9	34	54	48	0	0
10	35	127	138	2	0
11	36	33	50	0	0
12	37	106	16	0	0
13	38	35	83	0	0
14	39	140	24	0	0
15	40	35	66	0	0
16	41	68	58	0	0
17	42	30	52	0	0
18	43	70	51	0	0
19	44	147	66	0	0
20	45	18	94	2	0
21	46	108	78	0	0
22	47	38	80	0	0
23	48	85	63	0	0
24	49	48	42	0	0
25	50	61	71	0	0
Total		3260		5	
SEM		4.46		0.06	
Mean		65.2		0.10	
Test - control		65		0	

Table 2.3(c) An example of the raw data obtained with Peptostreptococcus species NCTC 9807.

Experiment 3.

Buccal cell number		Number of bacteria per buccal cell			
		test		control	
1	26	2	20	0	0
2	27	110	86	0	0
3	28	25	17	0	0
4	29	64	59	0	0
5	30	72	64	1	0
6	31	112	51	0	0
7	32	92	70	0	0
8	33	28	15	0	0
9	34	80	33	0	2
10	35	24	46	0	0
11	36	39	95	0	0
12	37	33	49	0	0
13	38	58	27	0	0
14	39	32	12	0	0
15	40	129	32	0	0
16	41	76	9	0	0
17	42	31	58	0	0
18	43	37	30	0	1
19	44	72	43	0	0
20	45	22	120	0	0
21	46	74	38	0	0
22	47	45	31	0	0
23	48	18	83	0	0
24	49	40	30	0	1
25	50	8	107	0	0
Total		2548		5	
SEM		4.52		0.05	
Mean		51.0		0.10	
Test - control		51		0	

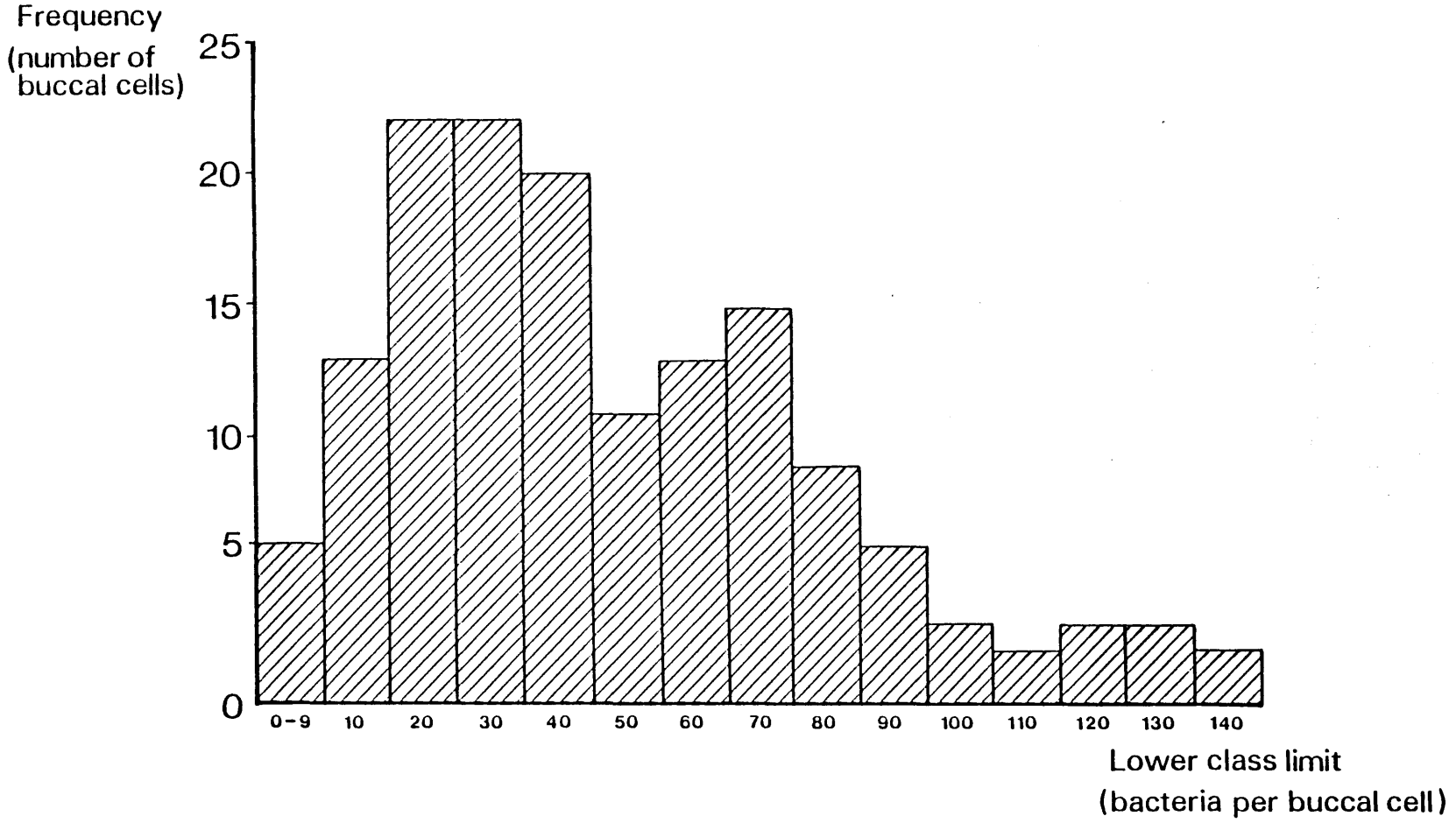


Figure 2.6 Frequency distribution of the numbers of Peptostreptococcus species NCTC 9807 adhering per buccal cell.

The results of all of the test bacteria are summarized in Table 2.4. The results showed wide variations in adherence between the different bacterial isolates studied, with results ranging from a mean of 0.4 to 83 bacteria per buccal cell. The Gram-negative rods, with the exception of the fresh strain B. gingivalis P4, adhered in relatively low numbers, from 1 to 8 bacteria per buccal cell. The Peptostreptococcus and Veillonella strains adhered in relatively high numbers, from 14 to 83 bacteria per buccal cell, as did the fresh strains of S. salivarius, S. sanguis and B. gingivalis. The type strains of the Streptococcus species and the fresh and type strains of A. israelii adhered poorly, 0.4 to 2.6 bacteria per buccal cell.

A comparison of the type and fresh strains demonstrated that with the B. gingivalis, B. intermedius, Veillonella species, S. salivarius, S. sanguis and A. israelii pairs, the fresh strains gave significantly increased adherence compared to the type strains ($p < 0.05$). The converse was true for the Peptostreptococcus species, where the type strain adhered significantly better than the fresh strain ($p < 0.05$). There was no significant difference between the adherence of the fresh and type strains of the Capnocytophaga ($p > 0.05$).

The coefficients of variation of the means ranged from 10 to 55 with an average of 29. However, where the numbers of bacteria adhering are small, the coefficient of variation will be inordinately large. Therefore, a more appropriate measure of experimental variance is obtained by considering means above a certain minimum value. This value was taken subjectively to be 10 or more bacteria per buccal cell and made comparisons with previous reports more standardized. Seven

Table 2.4 Bacterial adherence to buccal cells.

Bacterium	Number of bacteria per buccal cell				
	Experiment number				
	1	2	3	mean	± SEM
B. gingivalis P4	33	29	27	30	± 1.7
B. gingivalis W83	1.1	2.1	3.4	2.2	± 0.7
B. intermedius P2	2.0	5.7	5.6	4.4	± 1.2
B. intermedius 9336	1.2	1.2	0.5	1.0	± 0.2
Capnocytophaga sp. P2	4.8	3.0	2.6	3.5	± 0.7
Capnocytophaga sp. 27872	2.7	4.5	4.2	3.8	± 0.6
H. aphrophilus P5	8.4	5.6	9.9	8.0	± 1.3
H. actinomycetemc. 9710	7.3	6.7	8.6	7.5	± 0.6
Peptostrep. sp. P2	9.0	20	13	14	± 3.2
Peptostrep. sp. 9807	41	65	51	52	± 7.0
Veillonella sp. P3	86	92	72	83	± 5.9
Veillonella sp. 11463	21	24	17	21	± 2.0
A. israelii P2	2.4	1.4	1.3	1.7	± 0.3
A. israelii 10215	0.4	0.5	0.2	0.4	± 0.1
S. salivarius P2	41	37	31	36	± 2.9
S. salivarius 8618	1.9	3.9	1.9	2.6	± 0.7
S. sanguis P1	21	36	27	28	± 4.4
S. sanguis 7863	0.9	0.9	0.6	0.8	± 0.1

results had means of more than 10 bacteria per buccal cell, and these gave coefficients of variation of between 10 and 38, with an average of 21.

2.4 DISCUSSION

2.4.1 Problems with comparing adherence assays

Comparing the results of the present study with those of previous workers can be problematical due to a number of variable factors which cannot be easily quantified or controlled. Factors which contribute to these differences are: the source of buccal epithelial cells; the bacterial strains used; the concentrations of bacteria in suspensions; the types of buffer employed; and the staining techniques used to visualize and count attached bacteria. The following sections include a discussion of these different factors.

2.4.2 Buccal epithelial cells

The ability of exfoliated buccal epithelial cells to adsorb bacteria has been shown to vary from donor to donor and with time. For example, Gibbons and Dankers (1983) reported that, using a single strain of *S. sanguis*, the mean numbers of bacteria adhering to buccal cells from five donors were 13, 18, 25, 85 and 169. Likewise, Slots and Gibbons (1978) presented findings with *B. gingivalis* and three buccal cell donors, giving results of 81, 82 and 122 bacteria per buccal cell. Similarly, Isogai *et al.* (1986) found that *Bacteroides* species adhered poorly to gingival epithelial cells from normal rats, but adhered in high numbers to gingival cells from rats with naturally occuring gingivitis. Furthermore, it has been shown using non-oral epithelial cells that even with a single donor, although reasonable reproducibility could be achieved with replicate experiments on a

single day, considerable variation could occur on different days (Svandborg-Eden and Hansson, 1977; Isaacson et al., 1978).

Epithelial cells can be collected from various sites in the oral cavity. The easiest sites from which to obtain reasonable numbers of cells are the dorsum of the tongue and the buccal mucosa. However, cells from the former site usually harbour large numbers of attached bacteria which cannot be effectively removed, even after repeated washings, and can interfere with adherence experiments (Sklavanou and Germaine, 1980). Buccal cells obtained from the cheek mucosa, on the other hand, usually have comparatively few attached bacteria and have therefore been widely used (Gibbons and van Houte, 1971 and 1975; Okuda et al., 1981). Slots and Gibbons (1978), however, used buccal epithelial cells as well as cells collected from the gingival crevice. They tested the adherence of one strain of B. gingivalis with both cell types and obtained similar results; a finding which is notable, but cannot necessarily be assumed to be true for all bacteria.

The numbers of indigenous bacteria adhering to buccal cells have been shown to vary considerably from different individuals and from the same individuals at different times (Gibbons and van Houte, 1975). This was noticed in the present study, where the numbers of indigenous bacteria adherent to the control buccal cells ranged from a mean of 0.1 to > 20 bacteria per buccal cell, despite using a single donor (the author) throughout the experimental period and standardizing the collection procedure as far as possible. These contaminating bacteria may not only interfere with the counting of test bacteria, but may also influence their adherence. On several occasions throughout this study, buccal cell assays were discarded due to counts of greater than

two indigenous bacteria per buccal cell (Section 2.2.12).

Food debris may also contaminate buccal cells and alter their adherence properties. A normal diet will consist of innumerable chemicals, any of which may adsorb to and modify the buccal cell surface. For example, lectins from wheat germ or peanuts have been shown to affect the adherence of S. sanguis to buccal cells collected after consuming these foods (Gibbons and Dankers, 1983). Also, Samaha, Elvin-Lewis and Lewis (1986) noted that the adherence of S. sanguis to saliva treated hydroxyapatite was inhibited by tannin derived from tea, which could have a similar effect on the buccal mucosa. Therefore, in this study buccal cells were collected in the morning before any dietary intake for that day.

A number of salivary components have been shown to affect bacterial adherence (Stinson et al., 1982; Stanislawski et al., 1985; Cole, 1985) and buccal cells are naturally exposed to saliva in the oral cavity prior to collection. The variation of the flow rate and composition of saliva relative to the time of day, dietary stimuli, sleep, age, sex and drugs, are well documented (Mason and Chisholm, 1975). Therefore, the extent to which the buccal cell surfaces will be modified by salivary components may depend on a number of factors. These were standardized as far as possible in the present study by collecting cells from the same individual at a specific time of the day.

2.4.3 Bacterial strains

Discrepancies between the results of different adherence studies may be due to inherent variation between bacterial species and between different strains of a single species. This variance may also be due to changes in the bacterial surface components which mediate adherence resulting from the maintenance of the strains by repeated subculturing. Williams and Gibbons (1975) noted that strains of S. salivarius, S. sanquis and S. mitior, when maintained by laboratory transfer for several months, tended to lose their ability to attach to epithelial cells. Also, Orstavik and Orstavik (1982) reported that the ability of S. mutans to adhere to glass decreased with the number of succesive in vitro transfers. These observations with S. mutans were confirmed by Westergren and Olsson (1983), who also related a decrease in adherence with a loss of hydrophobicity.

Bacterial surface components, such as pili, capsules, lipolysaccharides and membrane vesicles, which can mediate adherence (Slots and Genco, 1984) may be necessary for an organism to survive in the oral cavity. In the protected environment of the laboratory pure culture, however, such factors may confer no selective advantage, and furthermore, may require the expenditure of valuable energy. Therefore, cells that produce these metabolically expensive components may be eliminated by uncoated, adherence-deficient mutants which can grow more efficiently under laboratory conditions (Costerton, Geesey and Cheng, 1978).

Type cultures of bacteria, by definition, have been maintained under laboratory conditions for very long periods. They can

therefore, be regarded as laboratory adapted strains, and thus, may have lost cell surface components related to adherence. Such strains are clearly not ideal for use in adherence studies which attempt to relate to the *in vivo* situation. However, they are readily available to all workers, are well characterized, and are consequently useful for comparing the results of experiments carried out by different research groups.

The possible advantages and disadvantages of using type strains were recognized in the present study. Therefore, for each species of bacterium studied, a type strain and a freshly isolated, freeze-dried strain were used. A previous study, noting that strains lost their ability to adhere to buccal cells after several months of laboratory transfer, isolated new strains as required during the course of the investigation (Williams and Gibbons, 1975).

A comparison of the eight pairs of fresh and type strains shows that: with six species, the fresh strains adhered significantly better ($p < 0.05$); the *Capnocytophaga* species showed no significant difference ($p > 0.05$); and the type strain of the *Peptostreptococcus* species adhered significantly better than the fresh strain ($p < 0.05$). It might be expected that fresh strains would always adhere better than type strains, and although this appears to be the predominant trend, exceptions may occur.

2.4.4 Experimental buffers

The main suspending medium used by previous workers in buccal cell adherence assays was phosphate buffered saline (PBS), a commonly

used experimental buffer. However, the components of PBS bear little resemblance to saliva and therefore, it is difficult to extrapolate reports using PBS to the *in vivo* situation. For this reason, in the present study, a suspending medium was prepared which resembled more closely the ionic constituents of saliva, namely, saliva ions buffer (SIB) (Appendix 8). A similar buffer has been used by previous workers and the buffer used here was based on the formulation described by Clark *et al.* (1978) and Appelbaum *et al.* (1979).

The substitution of PBS with SIB in this study could affect experimental results in several ways. SIB contains 1 mM calcium chloride, whereas PBS contains none. Rolla (1977) reported that calcium ions may enhance the adherence of bacteria to each other and to oral surfaces by means of calcium bridging via the acidic groups of those surfaces. This was confirmed by Yamazaki *et al.* (1981) who noted that *Eikenella corrodens* adherence to buccal epithelial cells was enhanced in the presence of calcium ions. However, in contrast, Eifert *et al.* (1984) reported that calcium chloride inhibited the adhesion of *S. sanguis* to saliva treated hydroxyapatite, but only with concentrations greater than 1 mM (SIB contains 1 mM). Therefore, with regard to these reports, it is difficult to estimate the effect of calcium chloride in SIB on the adhesion of the different strains studied.

Gibbons and co-workers (1983a) demonstrated that with low concentrations of *S. sanguis* (2×10^7 bacteria per ml), 200 or 1000 mM sodium chloride inhibited adherence to saliva-treated hydroxyapatite, but at high bacterial concentrations (5×10^9 bacteria per ml) this effect was not significant. Eifert *et al.* (1984) using 6×10^9

S. sanguis per ml reported that increasing concentrations of both sodium chloride and potassium chloride caused a decrease in adherence to saliva treated hydroxyapatite. Since SIB contains 50 mM potassium chloride, and PBS contains 150 mM sodium chloride, it is unlikely that the differences in the concentrations of these monovalent ions in the two buffers would produce significant differences in adherence, although SIB might be expected to give higher levels of adherence. Eifert and co-workers also reported that magnesium chloride inhibited adherence, but only with concentrations exceeding 2 mM and SIB contains only 0.1 mM magnesium chloride. They concluded that the optimum buffer for a hydroxyapatite adherence assay with S. sanguis should contain 2 mM phosphate (pH 6), 5 mM potassium chloride and 1 mM calcium chloride; constituents similar to those used in SIB. Although this formulation was devised for a hydroxyapatite adherence assay, certain similarities to the buccal cell system are evident, namely the presence of salivary components on the test surfaces and the use of oral bacteria.

These reports suggest that the composition of the buffer used in an adherence assay could markedly affect the subsequent attachment of bacteria, although little information is available on the effect of such buffers on the adhesion of the bacterial species used in this study. The choice of a buffer with ionic constituents similar to saliva is desirable, although the inclusion of the entire complex mixture of compounds found in saliva would be quite impracticable. Therefore, a compromise was reached in this study by using a buffer that contained the main ionic constituents of saliva, but was relatively simple in composition (Appendix 8).

2.4.5 Bacterial growth conditions

In addition to the effects of the suspending media on adherence, the conditions under which bacteria are grown may affect adhesion. For example, the adherence of S. sanguis, S. mutans and S. milleri to different surfaces has been shown to be affected by the growth medium employed, particularly with respect to the carbohydrate source (Wu-Yuan, Tai and Slade, 1979; Rosan et al., 1982a; Rogers et al., 1984).

The rate at which bacteria can grow may also affect the adhesive properties of bacteria. Growth rates depend largely on nutrient availability, and the supply of growth limiting nutrients can be controlled by using continuous culture techniques and altering the dilution rate of the system. For example, slow growing cells of S. mutans were found to be more hydrophobic (Rogers et al., 1984) and were more able to colonize the oral cavities of rats (van der Hoeven and Rogers, 1983) than fast growing cells. These properties could be related to the production of lipotechoic acid, which has been shown by Hardy et al. (1981) to be influenced by the growth rate of this organism.

In contrast, fast growing S. sanguis cells were shown to adhere to saliva treated hydroxyapatite comparably to batch grown cells, but better than slow growing cells which did not compete for binding sites and no longer demonstrated specific binding. However, these differences were only evident with glucose grown cells; bacteria grown slowly with fructose behaved like fast or batch grown cells (Rosan et al., 1982a).

The phase of growth reached by bacteria in batch culture may also affect adherence. For example, it has been reported that Actinomyces naeslundii (Saunders and Miller, 1980) and Acinetobacter calcoaceticus (Rosenberg et al., 1981) harvested at the stationary phase of growth adhered better to buccal cells than did bacteria collected at the early logarithmic phase.

Batch grown bacteria are generally used for adherence experiments, but organisms introduced into the oral cavity naturally will have been grown in vivo and may have quite different properties. Ideally bacteria used for experimental purposes should be grown under conditions most closely resembling the in vivo environment. However, the laboratory pure culture cannot possibly mimic the situation in the oral cavity. Nutrient rich media are required to grow the more fastidious bacteria and, outside of the use of complicated chemostat cultures, bacteria cannot be grown easily at the slow rates (mean generation times of 8 - 12 hours) thought to occur in the oral cavity (Gibbons, 1964). For practical reasons, therefore, cultures were grown in a simple standardized manner in batch cultures using complex media and were harvested in the early stationary phase of growth.

2.4.6 Bacterial concentrations

Adherence has been shown by a number of workers to be dependent on the concentration of bacteria available (Hillman, van Houte and Gibbons, 1970; van Houte and Green, 1974; Gibbons et al., 1983c and 1985a). Bacterial concentrations used in adherence assays therefore need to be standardized because this will affect the accuracy of the results obtained. The methods used by previous workers to standardize

bacterial concentrations in adherence assays have been limited to using established techniques. Reports usually refer to the use of standard counting chambers (Gibbons and van Houte, 1971; Appelbaum et al., 1979; Weerkamp and McBride, 1980a; Kagermeier and London, 1985), viable plate counts (Yamazaki et al., 1981), or more usually make no mention of the method used (Orstavik, Kraus and Henshaw, 1974; Slots and Gibbons, 1978; Rosenberg et al., 1981; Komiyama and Gibbons, 1984a).

During the inital stages of this study, a Thoma bacterial counting chamber (Weber Scientific Instruments Ltd., Lancing, England) was used, however several problems were encountered and the accuracy achieved was consequently poor. These inaccuracies resulted mainly from inadequate visualization of the bacteria in the counting chamber (even with stains added to the suspending medium) and from the bacteria forming an uneven distribution within the counting chamber. In addition, with this method each suspension on each occasion prior to use must be counted microscopically, the concentration of bacteria calculated and finally the suspension diluted as required. The assay results obtained will be dependent on the accuracy of the count performed on each occasion and it was felt that counts could not be achieved with the desired degree of accuracy or reproducibility using counting chambers.

Plate counts were also used in this study, but had the disadvantage of only determining viable counts of a bacterial suspensions, not total cell counts. Thus, with bacteria such as the anaerobes that may suffer a rapid loss of viability in experimental buffers, the viable counts may be well below the total cell counts and

a large degree of variance will be introduced.

A method of standardizing bacterial concentrations using a direct epifluorescence filter technique was therefore developed that reduced the inaccuracies encountered with conventional techniques. This method, based on a technique used to count bacteria in milk (Pettipher et al., 1980), involves adjusting the volume of a bacterial suspension to a pre-determined optical density to give a concentration of 10^8 bacteria per ml. Using this technique a suspension can be adjusted accurately and with ease in minutes with a high degree of accuracy in a reproducible manner. Also, the count is repeated three times and averaged, providing an optical density value which is used for all subsequent experiments with that organism, thus any error attributed to the initial count will be constant for all of the experiments performed with that organism. Furthermore, this technique was particularly appropriate for use in this study because the counts were performed in a similar manner to that used in the adherence assays, ie. the bacteria were viewed microscopically, deposited on a filter and stained with acridine orange.

2.4.7 Acridine orange staining

A selection of stains were tested in the early stages of this study for their ability to selectively stain bacteria adhering to buccal cells. The stains tested included those used by previous workers: crystal violet (Gibbons and van Houte, 1971; Slots and Gibbons, 1978; Okuda et al., 1981), gentian violet (Yamazaki et al., 1981), Giemsa (Hartley et al., 1978) and a combination of alcian blue and safranin (Reid and Brooks, 1982). In all cases, these stains

proved effective for visulizing Streptococcus species, but were unsatisfactory for counting Bacteroides species due to the poor contrast obtained against the buccal cells.

By comparison, acridine orange was found to be superior and was therefore used in this study. Small bacteria such as the Bacteroides species could be clearly distinguished from the buccal cells and contaminating debris. With larger bacteria such as the Streptococcus species it was possible to count adherent bacteria with ease. Also, divisions between adjoining cells were clearly visible which enabled the observer to decide objectively if a cell should be recorded as single or double, so reducing the possibility of biased counting.

Preliminary experiments indicated that the extent of staining of buccal cells and adherent bacteria was related to the pH of the staining solution. Acridine orange is essentially a cationic dye that will bind to acidic groups (phosphoric, carboxyl, etc.) of proteins by salt linkages (Michaelis, 1947). Such reactions are clearly affected by pH, which dictates the extent of dissociation of any acidic groups of the substrate, and consequently their availability to react. As the pH is reduced, less acidic groups will be dissociated, so less will be available for binding acridine orange molecules.

Another factor affecting staining with acridine orange is the metachromatic nature of the dye. Fluorescence metachromasia is due to dye molecules binding to adjacent acidic groups in close enough proximity to form dimers and polymers that fluoresce at a longer wavelength than monomer units. Acridine orange fluoresces green when in the orthochromatic form (monomers) and orange in the metachromatic

form (dimers and polymers). Hence, orange (metachromatic) fluorescence is indicative of a high local concentration of the dye. In general, green fluorescence results from staining DNA, which takes up acridine orange poorly; while orange fluorescence indicates the presence of RNA, denatured DNA or acidic polysaccharides (Rost, 1980).

The effect of these properties of acridine orange on the staining of buccal cells and bacteria was evident microscopically. At neutral or high pH, both buccal cells and bacteria stained very brightly, and there was insufficient contrast to clearly distinguish them. As the pH of the staining solution was decreased, the intensity of the fluorescence diminished. At pH 3.3 the buccal cells stained poorly, emitting a pale green fluorescence. Adherent bacteria, on the other hand, although staining less well than at high a pH, retained more of their fluorescence and contrasted clearly against the buccal cells.

The effect of metachromatic staining was most evident where bacterial cells of certain strains exhibited both green and orange fluorescence. This phenomenen has been advocated for the differentiation of living (green) and dead (orange) bacteria, but was subsequently found to be unreliable (Rost, 1980). Therefore, no attempt has been made to correlate the proportions of orange and green bacteria with bacterial viability in this study.

Contrast between adherent bacteria and buccal cells was further improved by quenching excess fluorescence with potassium permanganate. Ward and Fothergill (1976) demonstrated the quenching effect of potassium permanganate on fluorescence, and in this study it was used

succesfully at a concentration of 0.025 per cent. The quenching effect improved the contrast by reducing background fluorescence without substantially affecting bacterial fluorescence.

2.4.8 Experimental variance

One of the aims of this study was to develop a standardized adherence assay to provide accurate and reproducible results. To compare this study with previous studies coefficients of variation were calculated, however, the size of the bacterial population considered will influence the values obtained. The mean coefficient of variation of all the results in this study was 29, but a more realistic indication is obtained if only those results are considered that have, for example, ten or more bacteria per buccal cell, so that a reasonable population of bacteria is counted. The seven results within this category gave a mean coefficient of variation of 21. The effect of small sample size was more evident with previous reports, therefore, coefficients of variation calculated from the reports considered below also exclude results with means of less than 10 bacteria per buccal cell. Coefficients of variation are calculated from standard deviations, but most papers state the standard errors of the means and may not give the number of repeat assays, therefore it is only possible in some cases to determine the minimum coefficient of variation.

Yamazaki et al. (1981) and Saunders and Miller (1980) presented buccal cell adherence results, using Eikenella corrodens and A. naeslundii, with mean coefficients of variation of 14 and 17, respectively. However, these reports note that the results consist of

experiments performed in duplicate. Clearly, if experiments are repeated at the same time with the same materials, the results will appear to be highly reproducible. Such results do not indicate the reproducibility of experiments performed at different times with fresh bacterial cultures and buccal cells with the inherent levels of day to day experimental variation which has been reported by previous workers (Svandborg-Eden and Hansson, 1977). Slots and Gibbons (1978) carried out buccal cell adherence assays with a variety of oral bacteria on at least two occasions for each result and obtained a coefficient of variation of > 33. Gibbons and van Houte (1971) and Okuda *et al.* (1981), using oral streptococci and *B. gingivalis* respectively, reported data with mean coefficients of variation of > 22 and 24, respectively.

In this study experiments were carried out on three occasions on different days with fresh bacteria and buccal cells. Therefore, the careful standardization of the experimental procedures appears to have served its purpose in providing results which are, with respect to reproducibility, at least comparable to previously reported findings with all strains, and considerably better with others.

2.4.9 Comparisons of *in vivo* and *in vitro* results

The relationship between adherence and colonization has been noted in Section 1.2.1. If a bacterium is found to adhere to a particular surface, it is likely that it can be isolated from that surface *in vivo*; the converse is also true (Gibbons, 1984). Therefore, it is of interest to compare the results obtained in this *in vitro* adherence study with the reported incidence of the same

bacterial species *in vivo* from viable count techniques (Table 2.5, column 1). It should be noted that the *in vivo* incidences of bacteria are average estimates derived from a number of reports.

Bacteria cultured from the buccal mucosa *in vivo* will probably be derived from bacterial accumulations at other sites in the oral cavity, such as the tongue dorsum, the gingival crevice, or dental plaques. Some bacteria will be dislodged from these sites and suspended in saliva, and may subsequently adhere to the buccal mucosa. The extent of colonization in this case will depend on the concentration of bacteria in saliva and the affinity of the bacteria for buccal mucosal cells. In addition, while adherent bacteria may proliferate and increase in numbers, epithelial cell desquamation will tend to limit bacterial accumulation.

The apparent absence of cultivable bacteria of certain species on the buccal mucosa may be due to a number of reasons:

(i) The bacteria may be suspended in high concentrations in saliva but may be unable to adhere to buccal cells, or they may adhere in numbers too low for succesful recovery in culture studies.

(ii) The bacteria may not be present in saliva, or may be present in concentrations too low to allow sufficient numbers to adhere and thereby permit their recovery in culture studies.

(iii) The bacteria may adhere in recoverable numbers, but the laboratory techniques used may be unsuitable for isolation and identification of certain species of bacteria.

Table 2.5 Incidence of bacteria on buccal mucosa *in vivo* determined using cultural methods, compared with *in vitro* results from this and other studies (references 1 to 13 are listed in appendix 10).

Bacterium	Incidence on buccal mucosa *in vivo* from culture studies	*In vitro* adherence		
		Previous reports	Present study	
B. gingivalis	low[1]	high[6] moderate[7]	P4 W83	high low
B. intermedius	low[1]	moderate[6,7]	P2 9336	low low
Capnocytophaga sp.	low[2]	low[7]	P2 27872	low low
H. aphrophilus	low[2]	ND*	P5	moderate
H. actinomycetemc.	moderate[3]	ND	9710	moderate
Peptostrep. sp.	ND	ND	P2 9807	high high
Veillonella sp.	low[4]	ND	P3 11463	high high
A. israelii	ND	low[7]	P2 10215	low low
S. salivarius	high[2,5]	high[8-11]	P2 8618	high low
S. sanguis	high[2,5]	high[10-13]	P1 7863	high low

*ND - no data available.

All of the Gram-negative rods used in this study adhered poorly to buccal cells, except for the fresh B. gingivalis P4 strain. These organisms are found primarily in the gingival crevice, and only in small numbers on mucosal surfaces (Slots and Genco, 1984). This suggests that the absence of most of the Gram-negative rods on the buccal mucosa is due to the low affinity of these organisms. It is also possible that they are found in insufficient concentrations in saliva to be recovered from the buccal mucosa. This is suggested by the findings of Socransky and Manganiello (1971) that Gram-negative organisms are found only in low numbers in saliva. Also, some of these organisms are relatively fastidious and, even if present on the buccal mucosa in recoverable numbers, may not be successfully cultured.

The high affinity for buccal cells shown by B. gingivalis P4, indicates that some selective advantage may be conferred by this ability, possibly by adhering to epithelial cells in or near to the gingival crevice. This suggests that although the ability of oral Gram-negative rods to adhere to mucosal surfaces may not be essential for oral colonization, it may nevertheless be advantageous. However, it was shown by Slots and Gibbons (1978) that the adherence of a strain of B. gingivalis to buccal cells in phosphate buffered saline was inhibited if saliva was substituted as the suspending media. This may also be the case with B. gingivalis P4 in this study (although SIB was used), in which instance the high affinity demonstrated in vitro may not be expressed in vivo. However the assay procedures and bacterial strains used were quite different, and Slots and Gibbons made this observation with only one strain of B. gingivalis. Furthermore, Orstavik et al. (1974) noted that the adherence of

S. sanguis to bovine enamel was inhibited by suspending the bacteria in saliva instead of buffer in some experiments, but that adherence was enhanced in others. In order to overcome these inherent variations associated with different suspending media, saliva per se should ideally be used for in vitro adherence experiments. However, the use of saliva as a suspending medium may lead to other inconsistencies due to the intrinsic biological variations in salivary constituents relative to the time of day, dietary stimuli, sex and drugs (Mason and Chisholm, 1975). Therefore, it is likely that the problems encountered in the experimental use and collection of saliva have resulted in most researchers using buffers. In addition, the use of saliva may consequently interfere with comparisons with most previous studies.

Information on the oral distribution of the Peptostreptococcus species is limited, although data are available for the anaerobic Gram-positive cocci, of which the Peptostreptococcus species are major contributors. This group of organisms is found in the gingival crevice, in dental plaque, on the tongue and in saliva (Socransky and Manganiello, 1971), but no reports are available of their distribution on the buccal mucosa. In this study the Peptostreptococcus species demonstrated a high affinity for buccal cells which suggests that these bacteria may also colonize the buccal mucosa.

The Veillonella strains adhered well to buccal cells in this study and Veillonella species have been reported in high numbers in the gingival crevice, on the tongue dorsum and in saliva (Socransky and Manganiello, 1971; Gibbons and van Houte, 1975). However, they are found only in low numbers on the buccal mucosa in vivo (Liljemark

and Gibbons, 1971). This suggests that either the culture techniques used were unsatisfactory for these organisms or perhaps that Veillonella species adhere poorly when suspended in saliva in vivo.

Actinomyces species have been found in relatively high proportions on the buccal mucosa, on the tongue dorsum, in dental plaque and in saliva (Ellen, 1976). The Actinomyces species isolated by Ellen were divided into catalase positive and negative strains, which were refered to as A. viscosus and A. naeslundii, respectively. However, catalase negative oral Actinomyces species also include A. israelii, A. odontolyticus and A. meyeri (Holdeman et al., 1977). The A. viscosus strains were found in higher proportions on the tongue and in saliva, while the catalase negative strains were present in larger numbers on the buccal mucosa and in dental plaque. The A. israelii strains used in this study adhered poorly to buccal cells, which would suggest that these bacteria do not attach and colonize directly onto oral mucosal cells in vivo, contrary to the culture studies of Ellen (1976), although this study did not necessarily include A. israelii strains. However, it is feasible that saliva may promote the adherence of A. israelii to buccal cells in vivo. Alternatively, it is possible that the removal of bacterial aggregates from the A. israelii cultures prior to performing the assays may have eliminated the most adherent cells from the cultures.

Culture studies have shown that the Streptococcus species are the predominant bacteria found on the buccal mucosa. The results obtained in this study show that the fresh strains, but not the type strains, of both S. salivarius and S. sanguis adhere strongly to buccal cells. Therefore, while the results of the fresh strains

correlate well with the culture studies, clearly the type strains do not. The lack of adherence by the type strains is probably due to the loss of adherence conferring components as a result of laboratory maintenance. This illustrates the problem of using type cultures in adherence assays and confirms the findings of Williams and Gibbons (1975), Costerton et al (1978), Orstavik and Orstavik (1982) and Westergren and Olsson (1983).

2.4.10 Comparisons of in vitro results

Comparisons of the results from this study with those presented by other researchers are possible, although differences in experimental methods and bacterial strains may detract from the relevance of any conclusions that may be drawn. Even if the same methods are used, results may differ significantly. For example, although Slots and Gibbons (1978) and Okuda et al. (1981) used similar assay techniques to study the adherence of the same strain of B. gingivalis to buccal cells, the two groups reported quite different results.

The second column of Table 2.5 lists the results obtained by other researchers using in vitro buccal cell adherence assays, although the data presented are only estimates taken from the most appropriate previous reports. The results obtained in this study using fresh strains correlate in most cases, although the type strains produced results that were in most cases lower. Therefore, despite the differences in experimental methods and bacterial strains used, the results of this study are generally comparable to those of other reports.

2.5 Conclusions

One of the aims of this study was to develop and improve a technique for studying the adherence of various oral bacteria to exfoliated buccal epithelial cells. Numerous factors can affect adherence assay results unless great care is taken to control them. This study has attempted to do so, and thereby produce results which are as reproducible as possible. The methods developed were found to be simple and accurate and the standard errors of the means obtained for different experiments tended to be lower than those reported previously. The methods used are therefore recommended for the study of bacterial adherence to buccal epithelial cells *in vitro*.

The results show that half of the strains tested adhered well to buccal cells, however adherence did not correlate in all cases with the known distribution of the species *in vivo*. Therefore, the affinity of a bacterial strain for a particular surface *in vitro*, does not necessarily predict if the organism can colonize that surface *in vivo*.

The fresh strains gave results that correlated better than the type strains, with the *in vivo* cultural findings of other researchers. Therefore, the use of freshly isolated bacteria in adherence assays is recommended.

CHAPTER 3

AN *IN VITRO* METHOD TO STUDY THE ADHERENCE OF BACTERIA TO HELA CELLS

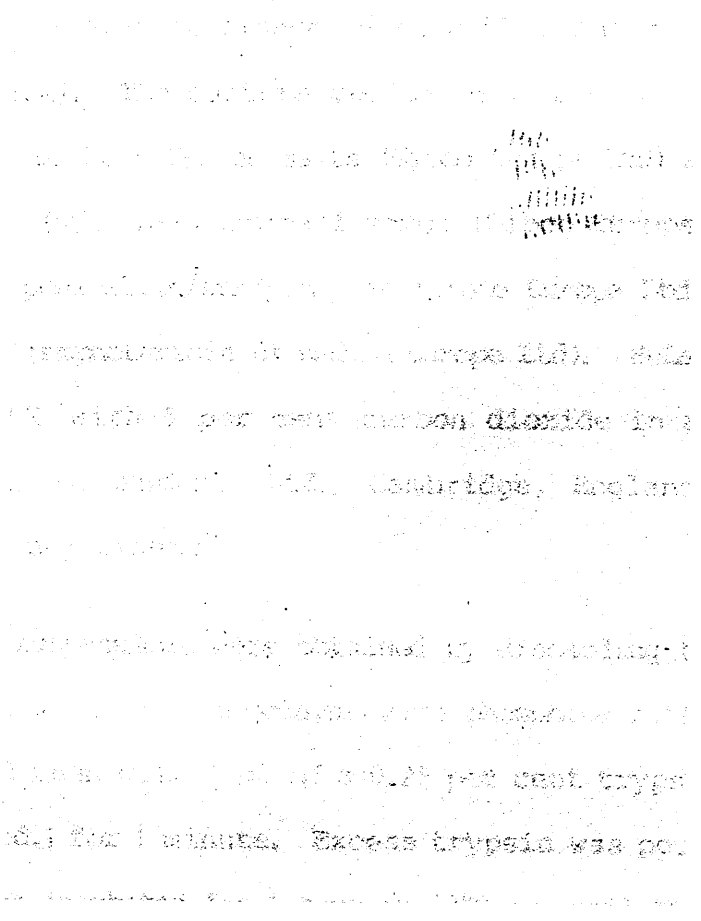
3.1 INTRODUCTION

The adherence of bacteria to the soft tissues of the oral cavity has been widely studied using exfoliated buccal epithelial cells. As discussed in Section 2.4.2, such cells create experimental problems with regard to contamination by indigenous bacteria, food debris and saliva, and variations in cell populations from different sites and different donors. Because of these inherent problems, a technique using standardized, laboratory cultured epithelial cell populations, free from contamination was developed.

A number of previous workers have used tissue culture cells for bacterial adherence experiments: Jones, Richardson and Uhlman (1981) studied the adherence and invasion of HeLa cells by *Salmonella typhimurium*; Sugarman and Epps (1982) studied the effect of oestrogens on the adherence of *Escherichia coli* and *Staphylococcus aureus* to HeLa cells; Pruzzo, Dainelli and Ricchetti (1984) studied the adherence of *Bacteroides fragilis* to Hela and intestine 407 cells; and Scaletsky *et al.* (1985) studied the adherence of different serotypes of *E. coli* to HeLa cells. Therefore, HeLa cells were chosen in the present study to obtain a standardized epithelial cell surface. However, it is noteworthy that no previous studies have used tissue culture cells to study the adhesion of oral bacteria. The technique which was adapted

for the present study was essentially that of Samaranayake and MacFarlane (1981), who used HeLa cells to investigate the adhesion of Candida albicans.

The bacteria studied were the same strains as used for the buccal cell assay in the previous chapter. The same staining technique was adopted because similar problems encountered with the staining and enumeration of bacteria on buccal cells also occured when HeLa cells were used.



3.2 MATERIALS AND METHODS

3.2.1 Preparation of bacterial suspensions

Suspensions of bacteria were prepared in saliva ions buffer (SIB) to a concentration of 10^8 bacteria per ml as described in Sections 2.2.1 to 2.2.8.

3.2.2 Maintenance of HeLa cells

HeLa cells were obtained from Gibco Europe Ltd. (Paisley, Scotland). The cells were maintained as monolayers in 80 cm^2/260 ml sterile disposable plastic tissue culture flasks (Nunc Inter Med., Kamstrup, Denmark). The culture medium used was Eagles minimum essential medium without Earles salts (Gibco Europe Ltd) supplemented with 10 per cent (v/v) newborn calf serum (Gibco Europe Ltd), 1000 units per ml of penicillin/streptomycin (Gibco Europe Ltd) and 2.5 μg per ml Fungizone (amphotericin B) (Gibco Europe Ltd). HeLa Cells were incubated at 37°C with 5 per cent carbon dioxide in a Grant CO_2 incubator (Grant Instruments Ltd., Cambridge, England) and were subcultured at 2 day intervals.

HeLa cell suspensions were obtained by discarding the nutrient medium and rinsing the cell monolayer with phosphate buffered saline (Appendix 9) and then with 1 ml of a 0.25 per cent trypsin solution (Gibco Europe Ltd.) for 1 minute. Excess trypsin was poured off and the monolayer was incubated for 1 hour at 37°C or until the HeLa cells became easily detached from the flask surface. The subsequent addition of 100 ml of fresh medium resulted in a uniform suspension of

HeLa cells. This suspension was then used to subculture the cells by adding 30 ml to a clean tissue culture flask, or to prepare monolayers on coverslips for adherence assays.

3.2.3 Preparation of HeLa cell monolayers on coverslips

HeLa cell monolayers were prepared on coverslips using a method based on that of Samaranayake and MacFarlane (1981) (Figure 3.1). Monolayers were grown on 22 x 22 mm glass microscope coverslips (Chance and Propper Ltd., Warley, England). Before use, the coverslips were cleaned by boiling in distilled water for 30 minutes. When dry, they were wrapped in tin foil and sterilised in a hot air oven at 170°C for 90 minutes. The coverslips were then placed singly in 35 mm diameter wells of sterile disposable plastic multiwell trays (Sterilin, Teddington, Middlesex, England) using aseptic techniques.

Hela cell monolayers were seeded onto the coverslips by adding 4 ml of HeLa cell suspension over the coverslips in the tissue culture tray wells. After 48 hours incubation the Hela cells formed confluent monolayers on the coverslips.

3.2.4 Treatment of HeLa cell monolayers

HeLa cell monolayers grown on coverslips were treated with saliva ions buffer (SIB), mixed saliva or serum, prior to the adherence experiments.

Whole mixed unstimulated saliva was collected from a healthy 25 year old dentate donor, blood group B rhesus negative, between 0930 and 1030 hours, before any dietary intake that day. Saliva was

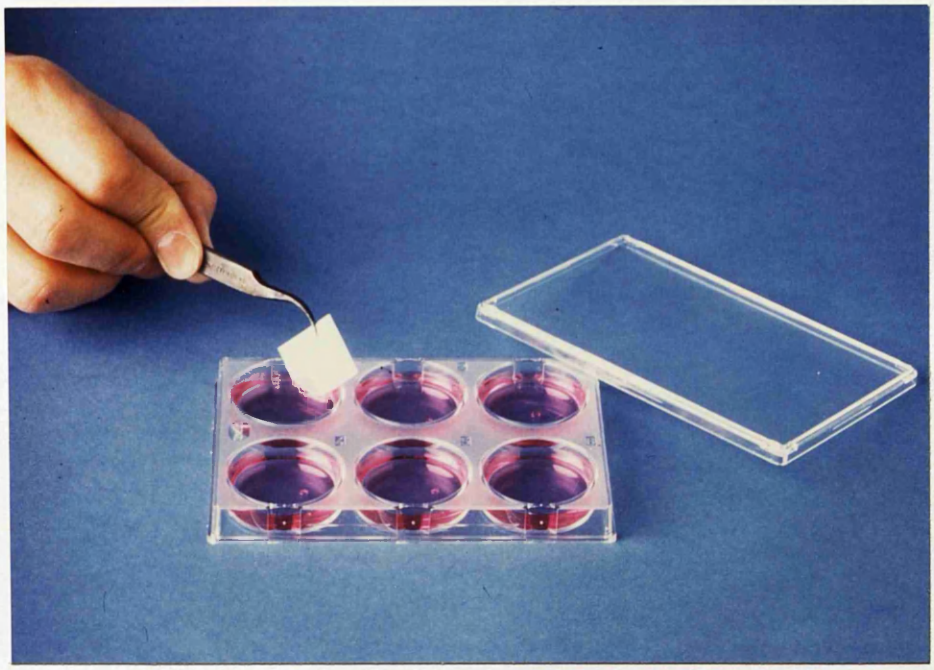


Figure 3.1 A tissue culture tray in which HeLa cell monolayers were grown on coverslips.

collected by expectorating into a sterile disposable universal (Nunc Inter Ltd.) held in ice. The saliva was clarified by centifuging at 40,000 g for 30 minutes at 4°C in an MSE high speed 19 centrifuge (MSE Scientific Instruments, Crawley, England). Prior to clarification, saliva was diluted with an equal volume of SIB at 4°C to prevent the significant loss of glycoprotein reported when undiluted saliva is centrifuged (Ericson, 1966). Saliva was always used on the day of collection and stored prior to use at 4°C.

Venous blood was collected from the same donor between 0930 and 1030 hours before any dietary intake. Serum was separated after the blood had been allowed to clot at 4°C by centrifuging at 3000 g for 10 minutes in an MSE super minor centrifuge (MSE Scientific Instruments). Approximately 50 ml of blood was collected at one time and the serum was dispensed in 5 ml aliquots into sterile disposable plastic bijoux (Sterilin) and stored for future use at -20°C. Serum was thawed immediately prior to use at 4°C.

To treat the HeLa cell monolayers, the nutrient medium was removed from the tissue culture wells with a glass pasteur pipette attached to a Venturi pump. The monolayers were then washed three times with 1.5 ml of SIB. Aliquots of 1.5 ml of either SIB, saliva or serum were then added to the wells to cover the monolayers which were then incubated for 10 minutes at 37°C in a rotary incubator (A. Gallenkamp and Co., London, England) at 60 r.p.m. After incubation, the monolayers were washed a further two times with 1.5 ml of SIB to remove unabsorbed saliva or serum components.

A treatment time of 10 minutes was used since initial experiments showed that Hela cells were sensitive to exposure to saliva and that periods of treatment greater than 10 minutes resulted in the monolayers detaching from the coverslips. When viewed microscopically the cells appeared to have rounded-up and resembled old or contaminated, dying cell populations. However, using an elipsometric technique with hydroxyapatite incubated in saliva, Ericson et al. (1982) noted that after 10 minutes 90 per cent of the adsorption of salivary components was completed compared with the uptake after 30 minutes. Although these results were obtained for hard tissues, assuming the adsorption of salivary components to HeLa cells occurs at a similar rate, it would be expected that only a small increase in adsorption would be obtained by increasing the exposure time.

3.2.5 HeLa cell adherence assay

After exposure of the HeLa cells to SIB, mixed saliva or serum, 3 ml aliquots of bacterial suspensions at a concentration of 10^8 cells per ml in SIB were added to the HeLa cell monolayers in the tissue culture wells. Monolayers incubated with SIB alone were used as controls to determine, if the HeLa cells were contaminated with bacteria, or any other form of debris which could affect the final counts. The trays were then incubated at 37°C for 60 minutes in a rotary incubator (Gallenkamp) at 60 r.p.m. Following incubation the monolayers were washed three times with 1.5 ml of SIB in the tissue culture trays to remove unattached bacteria, leaving Hela cell monolayers attached to coverslips with adherent bacteria.

3.2.6 Staining procedure

Immediately after washing, the monolayers were stained in the tissue culture tray wells with 1.5 ml of 0.025 per cent acridine orange (Hopkin and Williams Ltd., Chadwell Heath, Essex, England) in pH 3.3, 0.1 M citrate/sodium hydroxide buffer for 2 minutes, followed by two washes with 1.5 ml of SIB. The monolayers were then treated with 0.01 per cent potassium permanganate in distilled water for 2 minutes to quench background fluorescence and were given a further two washes with 1.5 ml of SIB. The coverslips with attached monolayers were then removed from the wells and air dried in a coverslip rack.

Before use, the acridine orange and potassium permanganate solutions were filtered through 0.45 μm pore size Sterifil D-HA filtration units (Nihon Millipore, Kogyo, Yonezawa, Japan) to ensure that no particulate matter was present, which could produce microscopic artifacts.

3.2.7 Bacterial enumeration

The dried, stained coverslips were mounted on 1.0 - 1.2 mm thick, 26 X 76 mm glass microscope slides with non-fluorescent Uvinert immersion oil (BDH Chemicals Ltd., Poole, England). The coverslips were placed on a drop of immersion oil on a microscope slide with the monolayer facing upwards with another glass coverslip mounted over the monolayer with another drop of immersion oil. The slides were then examined under an ultra-violet light at a magnification of X 1000 using a Nikon Optiphot microscope (Nippon Kogaku K.K., Tokyo, Japan).

The number of fields to be counted was determined using cumulative mean plots, as described in Section 2.2.12. Fifty fields were randomly selected by scanning horizontally and vertically over the coverslip, stopping approximately every 1 to 2 mm. The number of bacteria adhering to the monolayer in each field, equivalent to 0.018 mm^2, was then determined by eye. The diameter of the microscope field of view was determined using a stage micrometer graticule (Leitz, Germany). If any randomly selected field did not present an area of complete monolayer, the field was not counted and the slide was advanced to the next field.

3.2.8 Statistical analyses

The assay procedure was repeated a minimum of three times for each bacterium studied. The mean numbers of bacteria attached per microscope field (0.018 mm^2 at a magnification of X 1000), determined for each repeat assay, were averaged and the standard errors of the means and coefficients of variation were determined using standard statistical formulae.

The data obtained were shown to be non-parametric by graphical means as with the buccal cell results. The significance of differences between results were determined using the Mann-Whitney U test for comparisons between strains, or the Wilcoxon matched pairs signed-rank test for comparing differences between treatments.

This assay method was designed to be comparable to the buccal cell assay. However, the data from the buccal cell results were expressed as the mean number of bacteria per cell, while the HeLa cell

and tooth surface results (Chapter 4) were expressed as the mean number of bacteria per field of view under the microscope at a magnification of X 1000, an area equivalent to 0.018 mm^2. Therefore, the numbers of bacteria adhering per buccal cell were converted to the numbers of bacteria adhering per 0.018 mm^2 of buccal cell surface as follows.

The mean area of the buccal cells was determined using an Optomax System III image analyser (Micro Measurements Ltd., Saffron Walden, England) that used a closed circuit television scanning image analysis technique via the microscope. This system detects images by virtue of their contrast with the background and can give the percentage area of the field of view occupied by a buccal cell, from which its total area can be calculated. In addition to these data, a dilution factor was taken into account. The bacterial suspension was diluted 1 in 2 in the buccal cell assay by the addition of an equal volume of buccal cells to the bacterial suspension, a dilution not encountered in the other adherence assays. This information was then used to calculate the conversion factor of 8.51 by which the buccal cell results were multiplied.

3.3 RESULTS

All the bacterial strains under study were readily visualised and could be counted accurately. Staining of the HeLa cell monolayers and attached bacteria was comparable to that of the buccal cells, although the HeLa cell cytoplasm took up more acridine orange than that of the buccal cells. The nuclei of the HeLa cells are larger than those of the buccal cells in relation to the size of the cells, but the nuclei of both cell types stained to a similar extent (Figures 3.2 and 3.3).

The numbers of bacteria adhering to the HeLa cells varied considerably, ranging from 0.1 to 397 bacteria per 0.018 mm^2 of HeLa cell monolayer (Tables 3.1 to 3.4). The number of adherent bacteria depended on the strain of bacteria used and the treatment of the HeLa cells. Most strains adhered maximally to saliva treated HeLa cells (13 strains), the remaining isolates adhered maximally to the serum treated HeLa cells. All of the bacteria tested adhered least well to SIB treated HeLa cells.

Of the Gram-negative rods, the B. gingivalis and Haemophilus strains adhered in the largest numbers to HeLa cells treated with all three substrates. The highest affinities were shown by B. gingivalis P4 and H. actinomycetemcomitans NCTC 9710 to both saliva and serum treated HeLa cells. The Peptostreptococcus strains adhered well, with a high affinity demonstrated by the fresh strain for saliva treated HeLa cells, and by the type strain for all three substrates. Veillonella species P3 was the only other strain to adhere well to all three treated surfaces. Veillonella species NCTC 11463 adhered well

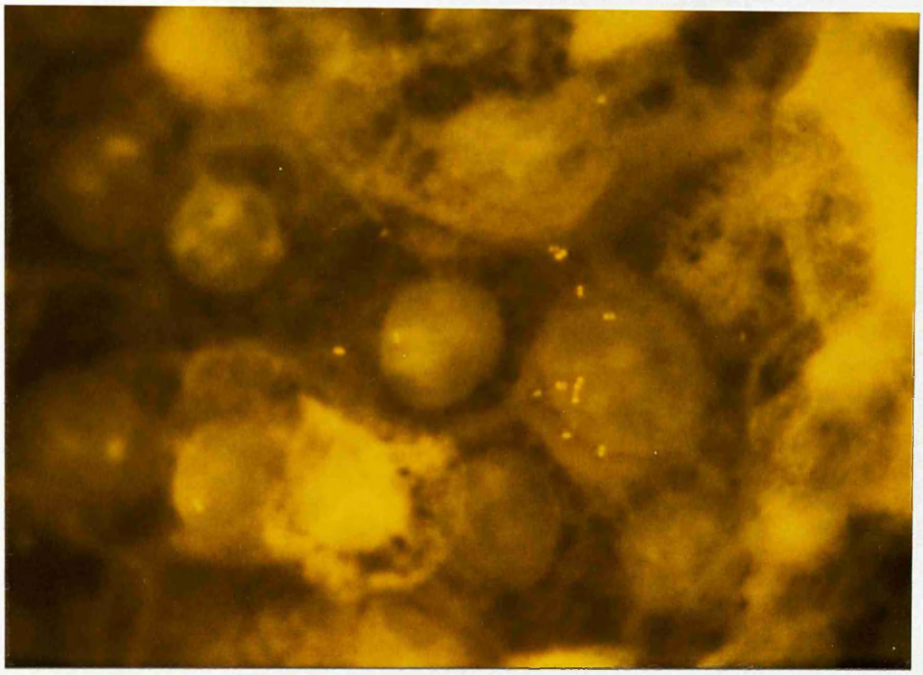


Figure 3.2 Peptostreptococcus species NCTC 9807 adhering to a HeLa cell monolayer. Magnification X 1000.

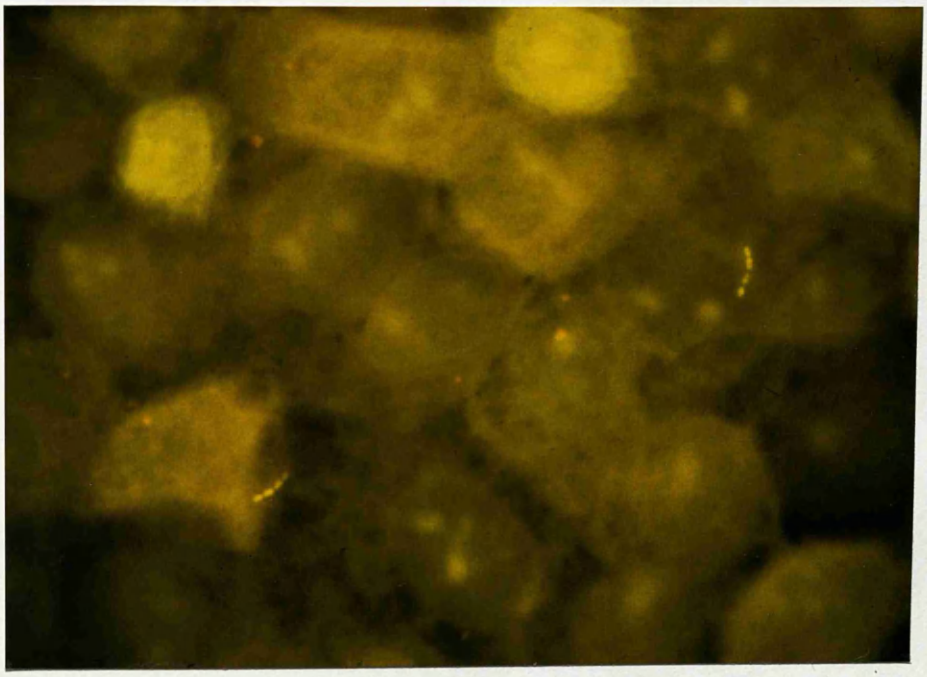


Figure 3.3 S. salivarius NCTC 8618 adhering to a HeLa cell monolayer. Magnification X 1000.

Table 3.1 Bacterial adherence to SIB treated HeLa cells.

Bacterium	Bacteria per 0.018 mm² of monolayer Experiment number 1	2	3	mean ± SEM
B. gingivalis P4	6.2	6.3	6.2	6.2 ± 0.03
B. gingivalis W83	9.0	17	14	13 ± 2.3
B. intermedius P2	0.4	0.7	1.0	0.7 ± 0.17
B. intermedius 9336	0.1	0.1	0.1	0.1 ± 0.0
Capnocytophaga sp. P2	2.3	4.0	4.0	3.4 ± 0.56
Capnocytophaga sp. 27872	0.8	0.6	1.2	0.9 ± 0.18
H. aphrophilus P5	4.8	4.2	7.8	5.6 ± 1.1
H. actinomycetemc. 9710	21	21	14	19 ± 2.3
Peptostrep. sp. P2	11	3.2	3.4	5.9 ± 2.6
Peptostrep. sp. 9807	262	178	208	216 ± 25
Veillonella sp. P3	147	100	114	120 ± 14
Veillonella sp. 11463	20	40	27	29 ± 5.9
A.israelii P2	2.5	1.8	2.6	2.3 ± 0.25
A.israelii 10215	0.5	0.1	0.1	0.2 ± 0.13
S. salivarius P2	17	20	15	17 ± 1.5
S. salivarius 8618	25	32	20	26 ± 3.5
S. sanguis P1	34	22	21	26 ± 4.2
S. sanguis 7863	23	16	20	20 ± 2.0

Table 3.2 Bacterial adherence to saliva treated HeLa cells.

Bacterium	Bacteria per 0.018 mm² of monolayer			
	Experiment number			
	1	2	3	mean ± SEM
B. gingivalis P4	112	91	98	100 ± 6.2
B. gingivalis W83	12	13	22	16 ± 3.2
B. intermedius P2	8.0	5.2	10	7.7 ± 1.4
B. intermedius 9336	0.1	0.1	0.1	0.1 ± 0
Capnocytophaga sp. P2	5.5	4.2	2.5	4.1 ± 0.89
Capnocytophaga sp. 27872	1.2	1.3	0.8	1.1 ± 0.15
H. aphrophilus P5	21	9.2	14	15 ± 3.4
H. actinomycetemc. 9710	130	81	69	93 ± 19
Peptostrep. sp. P2	133	139	108	127 ± 9.5
Peptostrep. sp. 9807	365	292	283	313 ± 26
Veillonella sp. P3	153	119	146	139 ± 10
Veillonella sp. 11463	72	98	93	88 ± 8.0
A. israelii P2	19	16	12	16 ± 2.0
A. israelii 10215	0.2	0.4	0.3	0.3 ± 0.06
S. salivarius P2	313	391	486	397 ± 50
S. salivarius 8618	483	345	148	325 ± 97
S. sanguis P1	297	234	273	268 ± 18
S. sanguis 7863	139	48	142	110 ± 31

Table 3.3 Bacterial adherence to serum treated HeLa cells.

Bacterium	Bacteria per 0.018 mm² of monolayer Experiment number 1	2	3	mean ± SEM
B. gingivalis P4	51	47	36	45 ± 4.5
B. gingivalis W83	10	34	40	28 ± 9.2
B. intermedius P2	1.0	0.5	0.9	0.8 ± 0.15
B. intermedius 9336	0.2	0.5	0.4	0.4 ± 0.09
Capnocytophaga sp. P2	1.6	0.8	1.0	1.1 ± 0.24
Capnocytophaga sp. 27872	0.3	0.6	0.7	0.5 ± 0.12
H.aphrophilus P5	27	25	18	23 ± 2.7
H.actinomycetemc. 9710	99	111	89	100 ± 6.4
Peptostrep. sp. P2	6.5	1.2	3.6	3.8 ± 1.5
Peptostrep. sp. 9807	168	181	155	168 ± 7.5
Veillonella sp. P3	308	234	262	268 ± 22
Veillonella sp. 11463	6.8	5.1	4.1	5.3 ± 0.79
A. israelii P2	3.7	1.5	2.4	2.5 ± 0.64
A. israelii 10215	0.0	0.2	0.1	0.1 ± 0.06
S. salivarius P2	5.4	2.0	4.6	4.0 ± 1.0
S. salivarius 8618	8.0	5.1	5.8	6.3 ± 0.87
S. sanguis P1	7.9	13	10	10 ± 1.5
S. sanguis 7863	20	4.5	5.0	10 ± 5.1

Table 3.4 Summary of bacterial adherence results to SIB, saliva and serum treated HeLa cells.

Bacterium	Bacteria per 0.018 mm² of HeLa cell monolayer treated with:		
	SIB	saliva	serum
B. gingivalis P4	6.2	100	45
B. gingivalis W83	13	16	28
B. intermedius P2	0.7	7.7	0.8
B. intermedius 9336	0.1	0.1	0.4
Capnocytophaga sp. P2	3.4	4.1	1.1
Capnocytophaga sp. 27872	0.9	1.1	0.5
H. aphrophilus P5	5.6	15	23
H. actinomycetemc. 9710	19	93	100
Peptostrep. sp. P2	5.9	127	3.8
Peptostrep. sp. 9807	216	313	168
Veillonella sp. P3	120	139	268
Veillonella sp. 11463	29	88	5.3
A. israelii P2	2.3	16	2.5
A. israelii 10215	0.2	0.3	0.1
S. salivarius P2	17	397	4.0
S. salivarius 8618	26	325	6.3
S. sanguis P1	26	268	10
S. sanguis 7863	20	110	10
MEAN	28	112	38

to only the saliva treated HeLa cells. Both A. israelii strains adhered poorly to all three surfaces. All four Streptococcus strains adhered well to saliva treated HeLa cells, but not to SIB or serum treated cells. Also, both S. salivarius strains adhered better to the saliva treated HeLa cells than either of the S. sanquis strains.

Distinctions between the adherence capacities of the fresh and type strains were evident on all three treated surfaces. The 8 pairs of bacteria tested (excluding the Haemophilus species) on the 3 surfaces gave 24 possible comparisons of fresh versus type strains. In 13 of these comparisons the fresh strains adhered better than the type strains ($p < 0.05$), while in 4 the converse was found ($p < 0.05$); in the remaining 7 comparisons there were no significant differences ($p > 0.05$).

The mean coefficients of variation for results of greater than 10 bacteria per 0.018 mm^2 of monolayer (as described for the buccal cell assay in Section 2.3.2) for SIB, saliva and serum treated HeLa cells were 24, 26 and 31, respectively, and were not significantly different. This suggests that the treatment of the HeLa cells has no or little effect on the reproducibility of the assay.

3.4 DISCUSSION

3.4.1 HeLa versus buccal cells

The aim of this method was to develop an alternative assay procedure for studying the adherence of oral bacteria to epithelial cells of human origin *in vitro*. Since the techniques used are very different to those used by other workers, a comparison of the results obtained using this method with previously reported results is of little value. The HeLa cell method was however, designed to be as similar as possible to the buccal cell assay described in the previous chapter to allow a comparison of both sets of results. Factors that were common to both assays were the bacterial strains, the growth media and conditions, the suspending medium (SIB) and bacterial concentration, the staining technique, and the incubation temperature and time.

HeLa cells are not, however, of oral origin and as such cannot be assumed to possess similar surface receptors and adherence properties as oral mucosal tissues. A comparison of the buccal and HeLa cell results shown in Table 3.5 indicates that, with the exception of *S. salivarius* NCTC 8618, the ability of the strains to adhere to SIB treated HeLa cells showed little resemblance to that found with buccal cells. These results contrast with those of Samaranayake and MacFarlane (1982) who demonstrated that *Candida albicans* grown in various carbohydrates adhered to PBS treated HeLa cells and to buccal cells in similar numbers.

Treatment of HeLa cells with clarified human, whole saliva, however, altered the adherence potential of the HeLa cells quite markedly. In every case adherence was increased; by as much as 23 fold with S. salivarius P2. A comparison of the results in Table 3.5 shows that of the three different treatments, saliva treated HeLa cells produced results most similar to those of the buccal cells. Furthermore, Table 3.6 shows that saliva treated HeLa cells are comparable with buccal cells in predicting the in vivo distribution of indigenous bacteria on buccal mucosa determined using cultural studies.

Treating HeLa cell monolayers with saliva or serum, may however, introduce additional sources of variance as a large number of factors can affect the composition of these biological fluids (Diem and Lentner, 1970; Mason and Chisholm, 1975). Unless care is taken to standardize the collection and treatment of saliva and serum, such additional variances may result. Therefore, these procedures were standardized as far as possible in the present study.

The mean coefficients of variation of the results (for values > or = 10 bacteria per 0.018 mm^2; Section 2.4.8) for the SIB, saliva and serum treated HeLa cells were 24, 26 and 31, respectively. These mean coefficients were not significantly different, indicating that treating the HeLa cells had little affect on the reproducibility of the assay. The buccal cell results gave a mean coefficient of variation of 21, which was also not significantly different from the HeLa cell results. Therefore, it appears that the HeLa cell assay yielded results that were as reproducible as those of the buccal cell method.

Table 3.5 Summary of bacterial adherence results to buccal cells (Table 2.4*) and SIB, saliva and serum treated HeLa cells (Table 3.4).

Bacterium	Bacteria per 0.018 mm² of cell surface			
	buccal cells	HeLa cells treated with: SIB	saliva	serum
B. gingivalis P4	252	6	100	45
B. gingivalis W83	19	13	16	28
B. intermedius P2	38	1	8	1
B. intermedius 9336	8	0	0	0
Capnocytophaga sp. P2	30	3	4	1
Capnocytophaga sp. 27872	32	1	1	1
H. aphrophilus P5	68	6	15	23
H. actinomycetemc. 9710	64	19	93	100
Peptostrep. sp. P2	119	6	127	4
Peptostrep. sp. 9807	445	216	313	168
Veillonella sp. P3	709	120	139	268
Veillonella sp. 11463	176	29	88	5
A. israelii P2	14	2	16	3
A. israelii 10215	3	0	0	0
S. salivarius P2	309	17	397	4
S. salivarius 8618	22	26	325	6
S. sanguis P1	238	26	268	10
S. sanguis 7863	7	20	110	10
MEAN	142	28	112	38

*Buccal cell results calculated as described in section 3.2.8.

Table 3.6 Incidence of bacteria on buccal mucosa *in vivo* determined using cultural methods, compared with *in vitro* adherence to buccal and saliva treated HeLa cells (references 1 to 5 are listed in appendix 10).

Bacterium	Incidence on buccal mucosa *in vivo* from culture studies	test strain	*In vitro* adherence	
			Buccal cells	saliva treated HeLa cells
B. gingivalis	low[1]	P4	high	high
		W83	low	low
B. intermedius	low[1]	P2	low	low
		9336	low	low
Capnocytophaga sp.	low[2]	P2	low	low
		27872	low	low
H. aphrophilus	low[2]	P5	moderate	low
H. actinomycetemc.	moderate[3]	9710	moderate	high
Peptostrep. sp.	ND*	P2	high	high
		9807	high	high
V. parvula	low[4]	P3	high	high
		11463	high	high
A. israelii	ND	P2	low	low
		10215	low	low
S. salivarius	high[2,5]	P2	high	high
		8618	low	high
S. sanguis	high[2,5]	P1	high	high
		7863	low	high

*ND - no data available.

HeLa cells can therefore be usefully employed to study the effect of various factors on adherence. For instance HeLa cells treated with factors such as serum, to mimic crevicular epithelial cells, could be used to yield comparable data from studies on the colonization of the gingival crevice. Similarly, HeLa cells may be treated with substances such as fibronectin, bacterial products, plant lectins, lipotechoic acid, enzymes, saliva or serum fractions, to study in detail parameters affecting adherence.

Overall, the use of HeLa cells was shown to have the following advantages over buccal cells for use in adherence assays:

(i) HeLa cells consist of uniform monolayers of epithelial cells derived from a single population.

(ii) The problem of contaminating oral bacteria adhering to buccal cells is eliminated.

(iii) HeLa cell monolayers can be grown on coverslips, are convenient to handle, and can be washed, stained and mounted easily.

(iv) They are readily available to researchers, so that studies done by different workers using these cells in a standardized assay may give comparable results.

3.4.2 Adherence to HeLa cells

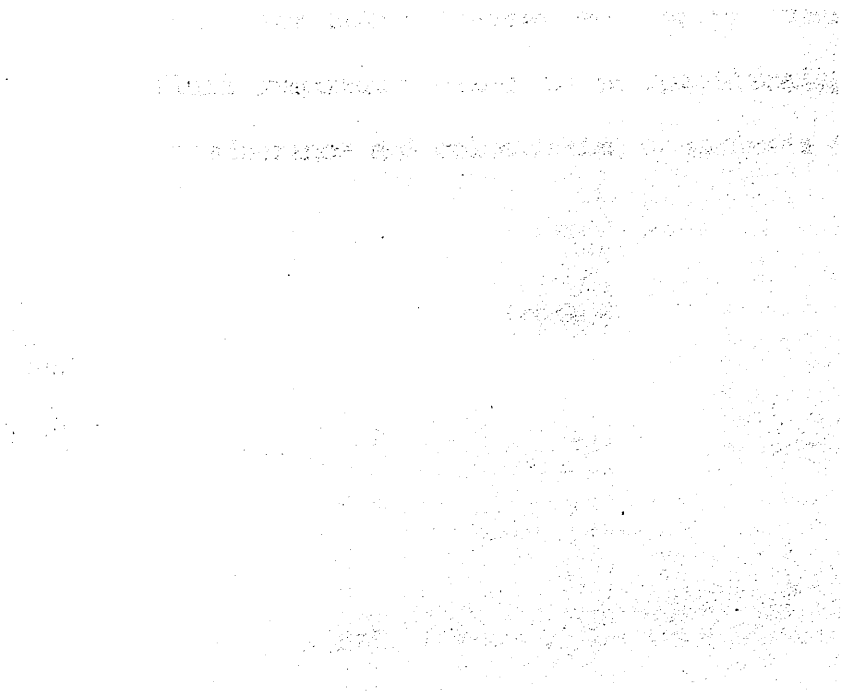
All of the bacteria that demonstrated a high affinity for buccal cells, namely B. gingivalis P4, H. aphrophilus P5, H. actinomycetem-comitans NCTC 9710, Peptostreptococcus species P2 and NCTC 9807, Veillonella species P3 and NCTC 11463, S. salivarius P2 and S. sanguis

P1, also showed a high affinity for saliva treated HeLa cells, with the exception of H. aphrophilus P5. However, an unusual result was noted with the two type strains of the Streptococcus species which adhered in high numbers to saliva treated HeLa cells, but not to the SIB or serum treated HeLa cells or buccal cells. HeLa cells may therefore adsorb a salivary component that enhances the adhesion of these two strains that is not adsorbed by or is easily washed off from the buccal cell surface. Such a factor may, of course, affect the adherence of the other bacteria used in this study, although this was not evident.

HeLa cells were treated with serum to mimic the in vivo environment where crevicular epithelial cells are exposed to crevicular fluid, which has a similar composition to serum (Diem and Lentner, 1970; Mason and Chisholm, 1975; Cimasoni, 1983). It can be theorized that bacteria in contact with such cells in vivo, may gain some selective advantage by adhering to them. Thus, the bacteria expected to adhere to serum treated HeLa cells would be the normal inhabitants of the gingival crevice. The bacteria shown to adhere well to serum treated HeLa cells were the B. gingivalis and Haemophilus strains, Peptostreptococcus species NCTC 9807 and Veillonella species P3. Indeed all of these species are found in the gingival crevice. Furthermore, the proportions of most of these bacteria have been reported to increase substantially in numbers with the progression of periodontal disease (Section 1.5.2) when crevicular fluid flow is increased (van Palenstein Helderman, 1981a). Therefore, it may be concluded that the ability to adhere to crevicular fluid coated epithelial cells may be an advantage to

certain members of the oral flora in selectively colonizing the gingival crevice.

The reduced adherence of the type cultures compared with the fresh isolates, evident in the buccal cell adhesion assay, was also found using HeLa cells. A decrease in the adherence of most of the type cultures was evident with all three treated surfaces. The results therefore indicate that the loss of adherence properties as a result of laboratory subculture is not specific for one kind of surface, but is a generalized loss of adherence to any test surface.



3.5 CONCLUSIONS

The HeLa cell adherence assay was demonstrated to be effective for measuring the adherence of oral bacteria to epithelial cell surfaces and has a number of advantages over the use of buccal cells. Furthermore, the use of saliva treated HeLa cells provides results comparable to those obtained with buccal cells. HeLa cells can also be treated with serum to mimic crevicular epithelial cells, or many other factors to enable adherence interactions to be studied further.

The use of saliva or serum to treat HeLa cells was shown to markedly influence the adherence properties of the epithelial cells. In general, most of the bacteria studied adhered in higher numbers to saliva treated HeLa cells, with the exception of a number of bacteria usually present in the gingival crevice area, some of which showed a higher affinity for serum treated HeLa cells. Thus, salivary and crevicular fluid components appear to be specifically implicated in the selective adherence and colonization of bacteria on oral surfaces.

CHAPTER 4

AN *IN VITRO* METHOD TO STUDY THE ADHERENCE OF BACTERIA TO SALIVA TREATED TOOTH ENAMEL

4.1 INTRODUCTION

Although the epithelial tissues constitute the largest surface area available for bacterial colonization within the mouth, the hard non-shedding surfaces of the teeth present a quite different and equally important surface for colonization. Generally, teeth harbour the largest masses of bacteria in the mouth, with dental plaques containing in the order of 10^{11} organisms per milligram of wet weight (Gibbons, 1964).

The association between dental plaque and dental diseases has produced much interest in the adherence of oral bacteria to teeth. Therefore, various methods have been developed to study bacterial adherence to a variety of hard surfaces (Gibbons, Etherden and Peros, 1985b). However, natural human tooth enamel has not been used widely due to problems in obtaining sufficient quantities in a suitable form for use in assays and because of difficulties in accurately quantifying adherent bacteria. As a result, the material most commonly used at present is synthetic hydroxyapatite in the form of small beads, in conjunction with a radioactive labelling assay technique (Clark *et al.*, 1978).

The use of natural tooth enamel is preferable when relating *in vitro* results to the *in vivo* environment because synthetic substitutes

may differ in their physico-chemical properties to natural enamel and may thereby significantly affect adherence reactions. Therefore, an assay method was developed which used sections of human tooth enamel, treated with saliva, to mimic as closely as practicably possible the in vivo environment. Enumeration of attached bacteria was by microscopical examination of sections stained with acridine orange using a similar protocol to that used in the buccal and HeLa cell assays, thus allowing valid comparisons to be made.

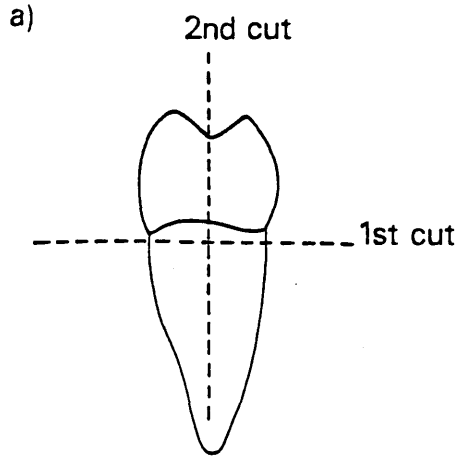
4.2 MATERIALS AND METHODS

4.2.1 Preparation of bacterial suspensions

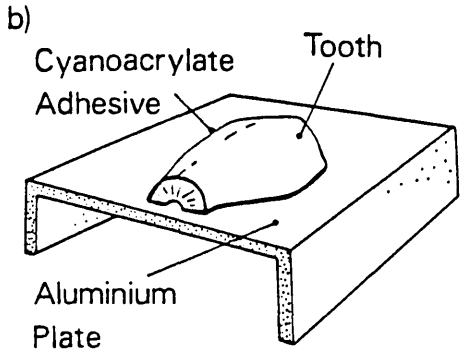
Suspensions of bacteria were prepared in saliva ions buffer (SIB) to a concentration of 10^8 bacteria / ml as described in Sections 2.2.1 to 2.2.8.

4.2.2 Preparation of tooth enamel pieces

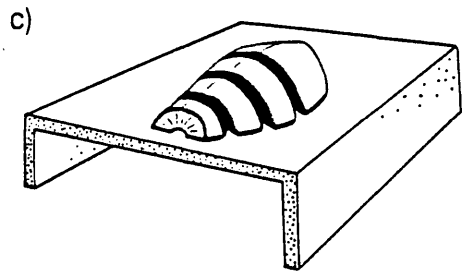
Healthy, clinically caries-free, premolar teeth that had been extracted for orthodontic reasons were obtained from various oral surgery units in the Glasgow area. Teeth were washed and stored prior to use in distilled water. The roots of the teeth were removed and the teeth were cut in half sagitally using a diamond impregnated wheel (J. and S. Davis Ltd., London, England) mounted in a dental hand piece (Kavo, West Germany). The flat, cut section was used to affix the teeth with cyanoacrylate adhesive (Loctite Ltd., Welwyn Garden City, England) onto an aluminium plate on which the teeth could be sectioned further. The sections were cut in the manner shown in Figure 4.1. After mounting on the aluminium plate, the tooth halves were cut laterally to give semi-circular sections which were broken off the mounting plate with ease using forceps. These were then split into two or three smaller pieces by breaking the sections between two pairs of forceps. This gave approximately twenty pieces from one tooth, with a clean cut enamel surface of at least $2mm^2$ per piece. The cut sides of the tooth pieces presenting the largest area of enamel were used for the adherence assays. The reverse side of each piece was marked with a proprietary red nail varnish so that the pieces could



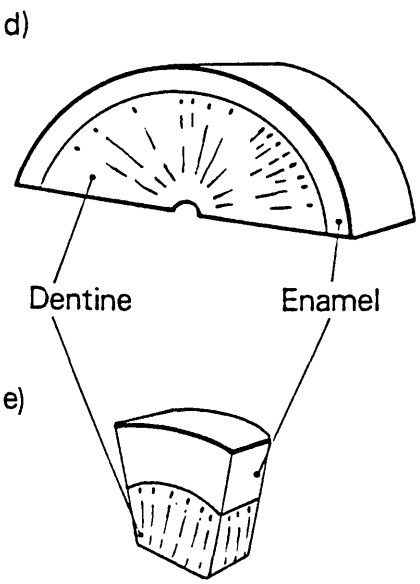
a) Healthy pre-molar, showing the first cut to remove the root and the second sagital cut to halve the crown.



b) One half of the tooth was mounted cut side down with cyanoacrylate adhesive onto a U-shaped aluminium plate.



c) Tooth was cut laterally to give three or four semi-circular sections.



d) Sections were broken off from the aluminium plate.

e) Each section was broken between two forceps to give two or three pieces. Adherence experiments were performed on a cut surface.

Figure 4.1 Preparation of tooth pieces.

easily be placed the correct way up for the adherence assay. The pieces were handled only with forceps to avoid grossly contaminating the enamel surface after cutting.

The tooth pieces were then placed in a sterile plastic disposable universal (Nunc Inter Med, Kamstrup, Denmark) and washed five times with 20 ml SIB, then left to equilibrate in SIB at 4°C for 24 hours.

4.2.3 Saliva treatment of tooth pieces

Prior to use the tooth pieces were washed twice in the universal with 20 ml of SIB, and the decanted SIB was replaced with 10 ml of clarified, mixed, unstimulated saliva, collected as described in Section 3.2.4. The universal was then placed horizontally in a rotary incubator (A. Gallenkamp and Co., London, England) at 37°C at a speed of 120 r.p.m. for 10 minutes. After incubation, the tooth pieces were washed three times in the universal with 20 ml SIB to remove unabsorbed salivary components.

4.2.4 Saliva treated tooth adherence assay

After treating with saliva, the tooth pieces were placed individually onto the bottom of sterile plastic bijou bottles, resting with the test surface upwards. Before the tooth pieces could begin to dry out, 2 ml aliquots of bacterial suspensions were added to the bijoux, care being taken not to turn the tooth pieces over. Control tooth pieces were incubated with 2 ml of SIB. The bijoux were then placed in a rotary incubator (A. Gallenkamp and Co.) at 37°C at a

speed of 60 r.p.m. for 60 minutes.

Following incubation, the bacterial suspensions were removed from the bijoux using a glass pasteur pipette attached to a Venturi pump. The tooth pieces in the bijoux were then washed three times with 2.5 ml of SIB.

4.2.5 Staining procedure

Immediately after washing, the tooth pieces were stained in the bijou bottles with 1 ml of 0.025 per cent acridine orange (Hopkin and Williams Ltd., Chadwell Heath, Essex, England) in pH 3.3, 0.1 M citrate/sodium hydroxide buffer for 2 minutes. The tooth pieces were then washed with two rinses of SIB (2.5 ml). Excess background fluorescence was then quenched with 2.5 ml of a 0.01 per cent solution of potassium permanganate in distilled water for 2 minutes, followed by a further two washes with 2.5 ml of SIB. The tooth pieces were then removed from the bijoux bottles with forceps and placed on a strip of filter paper to air dry. Before use the acridine orange and potassium permanganate solutions were filtered through 0.45 μm pore size Sterifil D-HA filtration units (Nihon Millipore, Kogyo, Yonezawa, Japan) to ensure that no particulate matter was present that could produce microscopic artifacts.

4.2.6 Bacterial enumeration

The dried, stained tooth pieces were mounted on 1.0 - 1.2 mm thick, 26 X 76 mm glass microscope slides with a small drop of cyanoacrylate adhesive (Figure 4.2). Care was taken to ensure that

Figure 4.2 Mounted tooth pieces.

the enamel surface was level and was not contaminated with adhesive. The tooth pieces were then covered with a drop of non-fluorescent Uvinert immersion oil (BDH Chemicals Ltd., Poole, England) and examined under ultra violet light at a magnification of X 1000 using a Nikon optiphot microscope (Nippon Kogaku K.K., Tokyo, Japan). The UV light passes from the source down through the objective lens, thus illuminating the tooth pieces from above.

Fifty fields were selected randomly by scanning horizontally and vertically over the enamel section of the tooth piece leaving approximately one field's width between counted fields. The number of bacteria adhering to the enamel surface in each field, equivalent to 0.018 mm^2, was then quantified visually.

4.2.7 Statistical analyses

This assay procedure was repeated a minimum of three times for each bacterium studied. The mean numbers of bacteria attached per microscope field (0.018 mm^2 at a magnification of X 1000) of enamel surface, determined for each repeat assay, were averaged and the standard errors of the means and coefficients of variation were determined using standard statistical formulae.

The data obtained was shown, by graphical means, to be non-parametric. The significance of differences between results were determined using the Mann-Whitney U test.

4.3 RESULTS

All of the bacteria under study were readily visualized and could be counted accurately. The enamel surface fluoresced a dull green, against which adherent bacteria contrasted adequately (Figures 4.3 and 4.4).

The numbers of adherent bacteria ranged from a mean of 0.2 to 584 bacteria per 0.018 mm^2 of enamel surface (Table 4.1). Of the Gram-negative rods, the fresh isolates of B. gingivalis and B. intermedius and the type culture of H. actinomycetemcomitans were found to adhere in comparitively high numbers. Also, both Veillonella strains, the type Peptostreptococcus strain and the fresh S. sanguis adhered in high numbers. None of the control enamel pieces incubated in sterile SIB demonstrated microscopically detectable levels of contaminating bacteria. The results gave a mean coefficient of variation of 20.

The fresh isolates adhered significantly better than the type cultures of the same species with six of the eight pairs ($p < 0.05$). The two Capnocytophaga strains adhered in similar numbers, and the Peptostreptococcus strains demonstrated maximal adherence by the type strain.

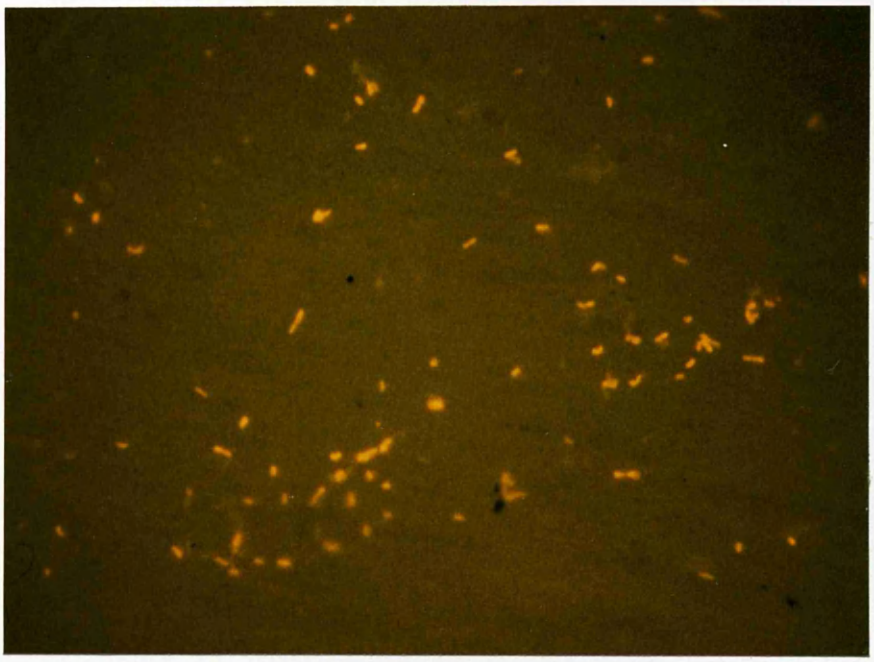


Figure 4.3 H. aphrophilus P5 adhering to a tooth section. Magnification X 1000.

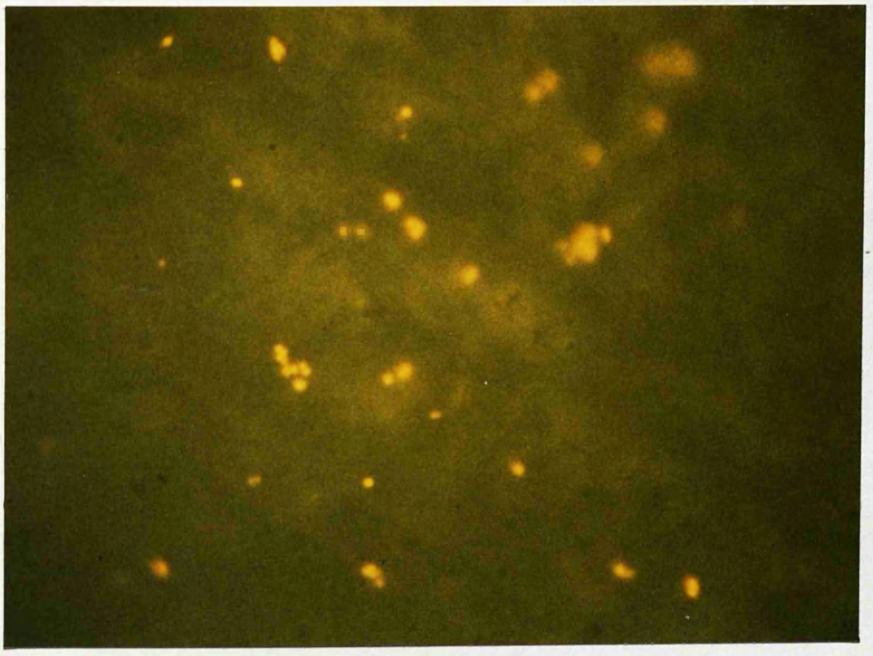


Figure 4.4 Peptostreptococcus P2 adhering to a tooth section. Magnification X 1000.

Table 4.1 Bacterial adherence to saliva treated tooth enamel.

Bacterium	Bacteria per 0.018 mm² of enamel Experiment number 1	2	3	mean ± SEM
B. gingivalis P4	118	167	98	128 ± 20
B. gingivalis W83	21	38	32	30 ± 5.0
B. intermedius P2	356	385	268	336 ± 35
B. intermedius 9336	10	2.9	8.2	7.0 ± 2.1
Capnocytophaga sp. P2	52	92	92	79 ± 13
Capnocytophaga sp. 27872	63	75	64	67 ± 3.8
H. aphrophilus P5	32	20	21	24 ± 3.8
H. actinomycetemc. 9710	327	330	314	324 ± 4.9
Peptostrep. sp. P2	47	35	42	41 ± 3.5
Peptostrep. sp. 9807	202	260	213	225 ± 18
Veillonella sp. P3	602	493	658	584 ± 48
Veillonella sp. 11463	125	188	139	151 ± 19
A. israelii P2	29	18	26	24 ± 3.3
A. israelii 10215	0.4	0.1	0.1	0.2 ± 0.1
S. salivarius P2	36	42	63	47 ± 8.2
S. salivarius 8618	29	14	22	22 ± 4.3
S. sanguis P1	237	172	182	197 ± 20
S. sanguis 7863	6.9	7.5	11	8.5 ± 1.3

4.4 DISCUSSION

4.4.1 Experimental method

Previously reported *in vitro* adherence studies related to oral bacteria have used a variety of hard surfaces of either artificial or natural origin. Powdered human enamel was used by Hillman *et al.* (1970) to study the adherence of plaque bacteria. However the powdered enamel was laborious to prepare and possessed a very large surface area. This made it very difficult to simulate the *in vivo* situation with respect to the ratio of bacterial numbers to enamel surface area. Synthetic hydroxyapatite powder was also used, being easier to produce, but possessed an even larger surface area to weight ratio (McGaughey, Field and Stowell, 1971). Other materials used have included slabs of bovine enamel (Orstavik *et al.*, 1974), glass tubes (Mukasa and Slade, 1973), whale dentine (Olsson and Krasse, 1976), discs of compressed hydroxyapatite (Clark and Gibbons, 1977), saliva treated glass (Stinson *et al.*,1981) and Mylar strips (Theilade *et al.*, 1982).

Clark and co-workers (1978) developed an adherence assay using hydroxyapatite beads that has since been widely used. These beads have a suitably small surface area to weight ratio, 0.63 square centimeters per milligram (Appelbaum *et al.*, 1979), and settle out of solution quickly so that unattached bacteria can be easily separated. However, X-ray diffraction analyses have shown that the beads consist predominantly of whitlockite rather than hydroxyapatite. Although whitlockite exhibits many similarities to hydroxyapatite with respect to bacterial binding sites (Gibbons *et al.*, 1985b), it is not ideal

for simulating the *in vivo* situation.

The use of natural tooth enamel surfaces is preferable because the interacting system will be closer to the *in vivo* situation. Adherence assays using human enamel have either used a powdered form (Hillman *et al.*, 1970), or else have used large sections of enamel in conjunction with laborious cultural procedures (Olsson and Krasse, 1976). The latter culture method involved removing adherent bacteria with increasingly severe washing techniques, culturing the washings, and finally layering nutrient agar onto the enamel sections to detect any remaining colony forming units of bacteria. Therefore, tooth sections have not been used widely probably because of a combination of difficulties involving obtaining a suitable number of enamel pieces with a clean surface and/or due to problems in quantifying adherent bacteria.

Using the method presented in this study, approximately twenty enamel pieces can be cut from a single premolar tooth. The pieces can be used immediately and do not require sterilisation since freshly exposed enamel is used which demonstrates microscopically undetectable levels of contaminating bacteria. Also, the pieces may be used after equilibration in buffer, or after treatment with saliva, serum or other factors.

A number of methods have been used to quantify adherent microorganisms; radiolabelling techniques (Clark and Gibbons, 1977), turbidimetric assays (Hillman *et al.*, 1970), cultural methods (McGaughey *et al.*, 1971; Olsson and Krasse, 1976) or microscopic examination (Orstavik *et al.*, 1974). Visual microscopic techniques

may be preferable as they permit the direct quantification of adherent bacteria. However, the problems associated with the clear visualisation of certain bacteria, as discussed previously for the assay methods using epithelial cells (Section 2.4.7), are also encountered with hard surface adherence assays. Also light will not pass through enamel blocks, and the preparation of large numbers of very thin translucent sections is impracticable. Therefore the epifluorescence technique used in the previous chapters was also employed in this assay. The ultra violet light passes from the source down through the objective lens and the staining method provided clear visualization of attached bacteria comparable to that obtained in the epithelial cell assays.

The cut enamel surfaces appeared on direct visual examination to be smooth and quite reflective. On microscopic examination the surfaces appeared smooth with occasional fine grooves running across the surface. In some cases, cracks in the enamel could be seen running across the surface into the section, but did not disrupt the integrity of the surface. The distribution of bacteria did not seem to be affected by these cracks or grooves. Certain areas of the enamel stained brighter than other areas for no apparent reason, but adherence was unaffected in these areas. The distribution of bacteria on the sections was generally uniform, although occasionally certain areas were seen to adsorb higher numbers of bacteria than others.

The mean coefficient of variation (for values > or = 10 bacteria per 0.018 mm^2; Section 2.4.8) of the tooth enamel adherence results was 20 per cent, which was not significantly different compared with the buccal and HeLa cell results ($p > 0.05$). This also compares

favourably with the mean coefficient of variation of 46 calculated for the results of Orstavik et al. (1974) who used a microscopic enumeration method to evaluate the adherence of streptococci to bovine enamel. However, saliva treated hydroxyapatite bead assays using radiolabelled bacteria appear to be generally more reproducible, with mean coefficients of variation of between approximately 3 and 30 (Gibbons and Etherden, 1982 and 1983; Gibbons et al., 1985a; Kagermeier and London, 1985). However, there are certain disadvantages associated with the use of radiolabelling techniques, for example measured radio-activity may be due to the adsorption of labelled bacterial or growth-medium components to the hydroxyapatite beads and not to the adherence of bacteria. With microscopic methods such errors are avoided since bacterial adherence is directly evaluated. Therefore, the advantage of greater reproducibility that it appears possible to obtain with radio-labelling techniques may be out-weighed by the introduction of additional experimental errors.

4.4.2 Comparison of in vitro results

The findings of other researchers studying the adherence of bacteria to tooth surfaces are listed in column 2 of Table 4.2 and are compared with the results obtained in this study. The method used to assess adherence in this study is quite dissimilar to the techniques generally utilised by other workers, most of which use hydroxyapatite beads and radiolabelled bacteria. In addition, the use of different strains of bacteria may produce widely different results. However, the innate affinities of different species for tooth surfaces may still be compared, so the following section includes a discussion of

Table 4.2 Incidence of bacterial species in dental plaque *in vivo* determined using cultural methods, compared with *in vitro* hard surface adherence results from this and other studies (references 1 to 37 are listed in appendix 11).

Bacterium	Incidence in supragingival plaque from culture studies	*In vitro* adherence to tooth surfaces	
		Previous studies	Present study
B. gingivalis	low[1-5]	high[26,27] low[22]	P4 high W83 low
B. intermedius	low[1-5]	high[27]	P2 high 9336 low
Capnocytophaga sp.	high[1,2]	low[23-25]	P2 moderate 27872 moderate
H. aphrophilus	low[2,5-8]	ND*	P5 low
H. actinomycetemc.	low[8,9]	high[20,27]	9710 high
Peptostrep. sp.	high[1-3]	ND	P2 low 9807 high
Veillonella sp.	high[1-4,10-13]	low[13]	P3 high 11463 high
A. israelii	high[1,2,13-15]	low[21]	P2 low 10215 low
S. salivarius	low[3,5,13,16-18]	low[27-30] moderate[25,31]	P2 low 8618 low
S. sanguis	high[5,13,16,19]	high[25-37]	P1 high 7863 low

*ND = no data available.

these comparisons.

A strain of B. gingivalis was shown to adhere in high numbers to saliva treated hydroxyapatite by Gibbons and Etherden in 1982 and 1983, but this strain was originally reported to adhere poorly by Slots and Gibbons in 1978. In each case similar assay procedures were performed using saliva treated hydroxyapatite beads, although the 1982 and 1983 papers used smaller assay volumes than the original method, and the 1983 paper used different bacterial concentrations. Such differences were taken into account when forming the estimates given in Table 4.2. The reasons for the discrepancies noted, could be due to changes in the experimental procedures or to alterations in the bacterial strain used due to laboratory storage over the four year period between the first and subsequent reports, although the latter possibility would more usually result in a decrease in adherence.

The results from this study showed that the fresh strain of B. gingivalis adhered well, but the type strain did not. Thus strain differences may cause markedly different adherence results with this organism, either due to inherently different affinities or to the loss of adherence confering components. Because of this latter possibility, the fresh strain is best considered as representative of this species and agrees with the later work of Gibbons group.

Gibbons and Etherden (1983) reported that B. intermedius possessed a high affinity for saliva treated hydroxyapatite, similar to the fresh strain used in this study with saliva treated tooth enamel. Thus, the results from this study using fresh strains of Bacteroides species agree with the findings of Gibbons group despite

the differences in the methods used.

Strains of the Capnocytophaga species were shown to adhere poorly to saliva treated hydroxyapatite beads by Celesk and London (1980) and Appelbaum et al. (1979). Also, Celesk, McCabe and London (1979) inoculated intact teeth suspended in nutrient broth with Capnocytophaga and found that although the bacteria colonized the cementum surface of the roots, the enamel surface remained free of bacterial plaques. Therefore, these three reports largely agree with the results of this study. However, it should be noted that the bacteria used by Celesk's group resembled the genus Capnocytophaga morphologically and biochemically, but lacked the carbon dioxide requirement characteristic of this genus and were therefore referred to as Cytophaga species.

No previous reports are available concerning the in vitro adherence of H. aphrophilus to enamel or hydroxyapatite surfaces. However, H. actinomycetemcomitans has been shown to adhere poorly to saliva treated hydroxyapatite beads (Gibbons and Etherden, 1983; Kagermeier and London, 1985), although this does not correlate with the H. actinomycetemcomitans type strain result from the present study. This discrepancy may be due to the H. actinomycetemcomitans strain possessing an atypically high affinity for the test surface. Alternatively, it is possible that the adherence properties of this strain may have been altered as a result of subculturing, although a loss of adherence would generally be expected under such conditions.

No previous studies have investigated the adherence of anaerobic streptococci to tooth surfaces, so the results obtained in this study

with the Peptostreptococcus species are novel findings and comparisons with other in vitro studies are not possible.

There are very few reports on the in vitro adherence of Veillonella species to teeth. The method most similar to the one used in this study was that of Liljemark and Gibbons (1971) using an in vivo assay. The procedure involved cleaning the labial surfaces of incisors and, after one hour, determining culturally if Veillonella, naturally present in saliva, had adhered to this surface. They found few, and concluded that this organism must have a low affinity for teeth. McBride and van der Hoeven (1981) came to a similar conclusion when they noted that Veillonella inoculated into gnotobiotic rats would not colonize in the absence of pre-formed streptococcal plaques. The findings of this study contradict these results, however the other methods used were poorly standardized and used quite different methods.

Most studies on the adherence of Actinomyces to tooth surfaces have been performed with either A. viscosus or A. naeslundii (Cisar et al., 1984). Only Clark et al. (1981) have used A. israelii, showing that it adhered poorly to saliva treated hydroxyapatite beads, which is therefore in agreement with the findings of this study.

S. salivarius was shown to adhere poorly to saliva treated hydroxyapatite beads (Gibbons and Etherden, 1983; Liljemark and Schauer, 1977; Clark et al., 1978) and to saliva treated enamel powder (van Houte et al., 1970). These results correlate with the findings of the present study. However, Appelbaum et al. (1979) reported that S. salivarius adhered moderately well to saliva treated hydroxyapatite

beads and Orstavik et al. (1974) reported the same using bovine enamel. Appelbaum used a method similar to that used in the other hydroxyapatite assays and therefore strain differences probably account for the different results. The use of bovine enamel instead of hydroxyapatite by Orstavik is another possible reason for the different results noted. The weight of the available data therefore tends to support the present findings which indicate that S. salivarius has a low affinity for human tooth enamel.

S. sanguis is the most widely studied organism used in this study and the 13 reports mentioned in Table 4.2 all noted that S. sanguis showed a high affinity for saliva treated hydroxyapatite beads (Liljemark and Schauer 1977; Clark et al., 1978; Liljemark et al., 1979; Appelbaum et al., 1979; Liljemark and Bloomquist, 1981; Nesbitt et al., 1982b; Gibbons and Etherden, 1982 and 1983; Gibbons et al., 1983c and 1985a; Eifert et al., 1984), saliva treated enamel powder (van Houte et al., 1970) or bovine enamel (Orstavik et al., 1974). These reports therefore correlate with the adherence of the fresh strain of S. sanguis in this study, but not with the type strain. The high affinity of S. sanguis for tooth enamel is not surprising as it one of the pioneer colonizers of the tooth surface in vivo observed in the initial phase of plaque formation (Doyle, Nesbitt and Taylor, 1982)

4.4.3 Fresh versus type strains

Freshly isolated bacteria were shown to adhere to saliva treated enamel better than the type strains in all cases, with the exception of the Peptostreptococcus species. The probable reasons for the

higher affinities of fresh isolates for various surfaces are explained in Section 2.4.3. The poor adherence of the fresh strain of the Peptostreptococcus species compared to the type strain may be due to one or more of the following reasons:

(i) Anaerobic streptococci are difficult to characterize and it is possible that if detailed taxonomic studies were carried out on the strains of Peptostreptococcus used, they might be assigned to different species. It is also possible that the fresh and type strains are the same species, but are different serotypes or biotypes with distinct surface receptors.

(ii) The fresh strain may simply adhere poorly in vivo. This may result in the elimination of this strain from the oral cavity, or it may, for example, be able to seek refuge in the protected environment of the gingival crevice where it may survive without adhering, or by aggregating with other bacteria.

(iii) The fresh strain may have lost certain adherence confering components within one or two sub-cultures after isolation. In support of this theory it was noted by Slots (1982a) that H. actinomycetmcomitans, on primary isolation on a selective agar medium, strongly adhered to the agar surface and commonly exhibited a colonial morphology with a star-like inner structure. However, both of these characteristics were rapidly lost within a few sub-cultures. Umemoto et al. (1986) reported that rough colony morphologies of H. actinomycetemcomitans correlated with the possession of fimbriae which were lost on subculturing.

(iv) The type strain selected may have a particularly high affinity for oral surfaces that may be mediated by components which cannot be lost as a result of laboratory sub-culturing; for example, essential cell wall components.

(v) The high affinity components of the cell walls of freshly isolated bacteria may be covered by surface structures with low affinities for oral surfaces, thereby preventing the former from participating in adherence reactions. On repeated sub-culture, the outer surface structures may be lost, exposing the underlying high affinity components. The loss of the outer surface structures in vivo may not occur if they offer some selective advantage over other competing oral flora. For example, Svandborg-Eden and Hansson (1978) reported that capsulated strains of Escherichia coli could only adhere to epithelial cells if their fimbriae extended outside of their protective capsule structure.

Although saliva treated enamel is a quite different surface from that of the soft tissues already studied, statistical analyses showed that the fresh Peptostreptococcus strains adhered significantly better ($p < 0.05$) than the type strains on all five kinds of surface studied (Table 4.3). Furthermore, the fresh strains of the remaining bacteria adhered significantly better ($p < 0.05$) or showed no significant difference compared with the type strains of the same species in 34 of the 35 remaining comparisons (the only exception was B. gingivalis on SIB treated HeLa cells). Considering that the different strains of bacteria showed marked quantitative variations in their affinities for the different surfaces, the consistent nature of the increased

Table 4.3 Summary of bacterial adherence results to buccal cells (Table 2.4[x]), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1).

Bacterium	Bacteria per 0.018 mm² of surface				
	buccal cells	HeLa cells SIB	HeLa cells saliva	HeLa cells serum	tooth enamel
B. gingivalis P4	252	61	100	45	128
B. gingivalis W83	19	13	16	28	30
B. intermedius P2	38	1	8	1	336
B. intermedius 9336	8	0	0	0	8
Capnocytophaga sp. P2	30	3	4	1	79
Capnocytophaga sp. 27872	32	1	1	1	67
H. aphrophilus P5	68	6	15	23	24
H. actinomycetemc. 9710	64	19	93	100	324
Peptostrep. sp. P2	119	6	127	4	41
Peptostrep. sp. 9807	445	216	313	168	225
Veillonella sp. P3	709	120	139	268	584
Veillonella sp. 11463	176	29	88	5	151
A. israelii P2	14	2	16	3	24
A. israelii 10215	3	0	0	0	0
S. salivarius P2	309	17	397	4	47
S. salivarius 8618	22	26	325	6	22
S. sanguis P1	238	26	268	10	197
S. sanguis 7863	7	20	110	10	8
MEAN	142	28	112	38	127

[x]Buccal cell results calculated as described in section 3.2.8.

adherence of one strain of a species compared to the other is quite notable. Therefore, it appears that the loss of adherence properties is of a generalised nature, as distinct from the loss of specific binding sites for particular surfaces.

4.4.4 Comparisons of *in vivo* and *in vitro* results

Adherence of bacteria to tooth surfaces *in vivo* may be modified by both saliva and previously attached bacteria. A bacterium which is unable to adhere to a bacteria-free tooth pellicle may still colonize the dentition by adhering to organisms already attached to the enamel surface. Alternatively, some bacteria may colonize a tooth by becoming mechanically trapped in retentive areas. Also, even if bacteria can adhere to tooth surfaces, other selective pressures may inhibit subsequent proliferation. Therefore, cultural studies indicating the presence of a particular bacterial species on the tooth surface (usually in plaque) may not necessarily correlate with its ability to adhere to clean tooth surfaces *in vitro*.

The data presented in column one of Table 4.2 (page 177) lists the approximate proportions of the various bacteria studied found in dental plaque using cultural techniques. The data obtained in this study shows that the results may depend on the particular strain of a species that is used, and that the fresh strains would be expected to give results more comparable to the *in vivo* situation. Although the type *Bacteroides* species adhered poorly to enamel *in vitro*, the fresh strain adhered well. These species exist in low numbers in supragingival plaque (Gibbons *et al.*, 1964b; Loesche and Syed, 1973; Moore *et al.*, 1982b and 1983; Liljemark, Fenner and Bloomquist, 1986),

but are commonly found in the gingival crevice (Takazoe et al., 1984). Therefore, it is feasible that the ability to adhere to enamel near the gingival crevice may assist in the colonization of these organisms, which may explain the high affinity demonstrated in this study.

Capnocytophaga species, in contrast to Bacteroides species, have been found in reasonable proportions in supragingival plaque (Moore et al., 1982b and 1983), but were found to adhere only moderately well to enamel. Therefore, the adherence properties of these strains appear to be less important in determining their colonization in vivo than other factors such as their ability to proliferate within the environment of supragingival plaque.

H. aphrophilus and H. actinomycetemcomitans have been found in low numbers in supragingival plaque (Kilian and Schiott, 1975; Kilian et al., 1976; Slots et al., 1980; Moore et al., 1983; Liljemark et al., 1984 and 1986). The present study concurs with the in vivo findings with H. aphrophilus, indicating that this organism adheres poorly to tooth enamel. On the other hand, H. actinomycetemcomitans adhered well in vitro. Therefore, the results obtained with these two strains indicate that the ability to adhere to enamel is not directly related to their presence in supragingival plaque.

Supragingival plaque has been reported to contain large proportions of Peptostreptococcus species (Gibbons et al., 1964b; Moore et al., 1982b and 1983), and the gingival crevice has similarly been reported to harbour large numbers of anaerobic streptococci (Gibbons et al., 1963). Therefore, these organisms might be expected

to adhere well to tooth surfaces, as was the case with the type culture but not the fresh strain. The unexpectedly low affinity of the fresh strain may be due to one of the reasons suggested in Section 4.4.3.

Veillonella species have been found in high proportions in dental plaque (Gibbons et al., 1964b; Slack and Bowden, 1965; Ritz, 1967; Liljemark and Gibbons, 1971; Loesche and Syed, 1973; Bowden, Hardie and Slack, 1975; Williams et al., 1976; Moore et al., 1982b and 1983). Since the strains tested in this study demonstrated a high affinity for tooth enamel, these results correlate well. In contrast, A. israelii has also been found in large numbers in dental plaque (Howell, Rizzo and Paul, 1965; Bowden et al., 1975; Loesche and Syed, 1978; Moore et al., 1982b and 1983), but both strains were found to adhere poorly to enamel in this study. One reason for the poor adherence of these strains could be the removal of the most adherent bacterial cells due to the separation of cell aggregates in preparing the test suspensions. More probably, A. israelii may colonize dental plaque without adhering to teeth, by aggregating with pre-existing dental plaque microorganisms (Slots and Gibbons, 1978).

The fresh strains of the Streptococcus species correlate well with the in vivo culture studies of other researchers. S. sanguis has been reported to be one of the earliest colonizers of cleaned tooth surfaces in the oral cavity (Doyle et al., 1982) and is a major constituent of dental plaque (Carlsson, 1967; De Stoppelaar, van Houte and Dirks, 1969; Bowden et al., 1975; Liljemark et al., 1986). This organism should therefore be able to adhere to saliva treated enamel, as confirmed by the results of this study. Conversely, S. salivarius

is found in very low proportions on teeth (Krasse, 1954; Gibbons, Kapsimalis and Socransky, 1964a; Gibbons et al., 1964b; Carlsson, 1967; Bowden et al., 1975; Liljemark et al., 1986) and was shown to adhere poorly to tooth enamel.

4.4.5 Buccal, HeLa and tooth adherence results

Comparisons of the tooth adherence results with the buccal and HeLa cell results, shows that there are wide variations in the abilities of the different strains to adhere to different surfaces. In general, however, the mean numbers of bacteria adhering to the different saliva treated surfaces, namely buccal cells, saliva treated HeLa cells and saliva treated enamel, are similar (Table 4.3 and Figure 4.5). The proportional distribution of bacteria on each of these surfaces though is quite varied. Certain strains adhere in similar numbers to all three surfaces and others show no similarities. Theoretically, certain similarities would be expected because of the presence of adsorbed salivary components on these surfaces. Conversely, the very different nature of the surfaces would predict quite different adherence patterns and the selective adsorption of different salivary components. The present results suggest that both phenomena may occur to varying degrees and that the eventual outcome will depend on these factors as well as the bacterial cell surface characteristics.

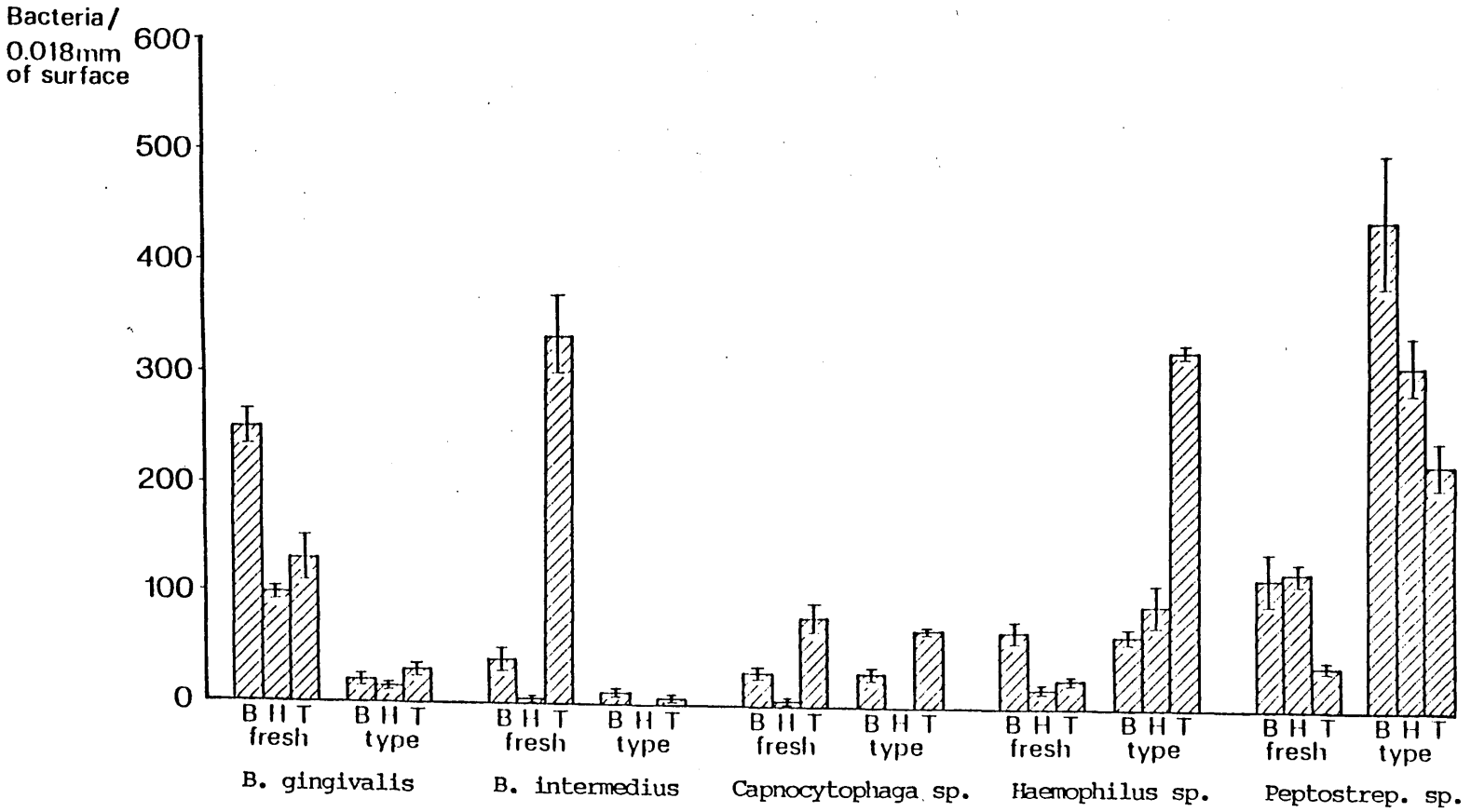


Figure 4.5 Comparisons of bacterial adherence to buccal cells (B), saliva treated HeLa cells (H) and saliva treated tooth enamel (T).

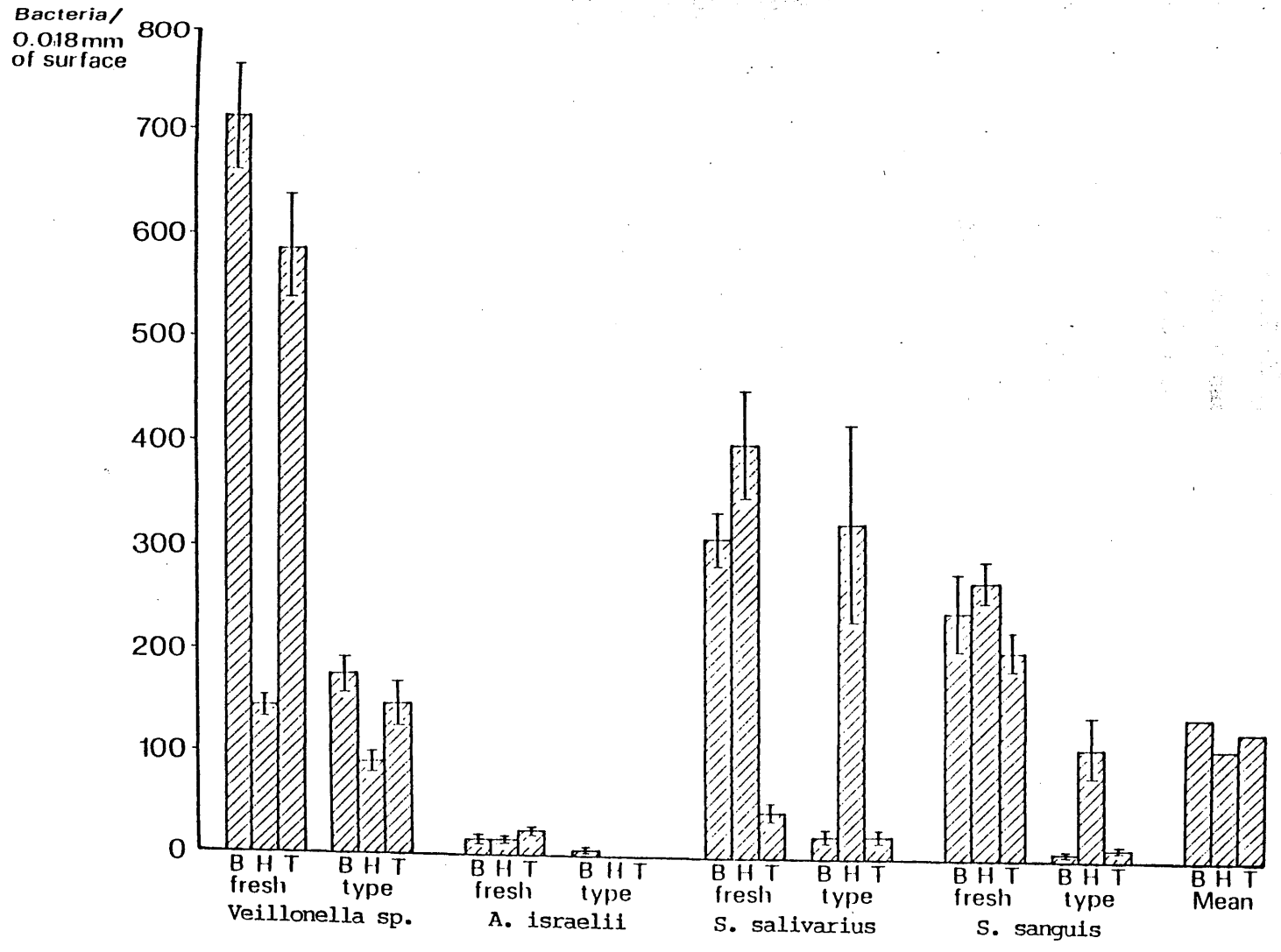


Figure 4.5 (continued).

4.5 CONCLUSIONS

The method presented proved to be simple to use and provided a relatively accurate means of quantifying bacterial adherence to saliva treated enamel *in vitro*. This method provides a useful means of studying adherence to natural tooth surfaces that is closer to the *in vivo* situation than the use of synthetic hydroxyapatite. In general the results obtained in this study agreed with the data from previously published reports.

The different bacterial strains varied markedly in their affinities for tooth enamel, as well as for buccal and HeLa cells. Despite this variance, the results showed that differences between the affinities of fresh and type cultures were fairly constant regardless of the test surface. This suggests that the loss of adherence properties by type strains is of a generalized nature, as distinct from the loss of specific binding sites.

The results of this study did not correlate in all cases with the results from *in vivo* cultural studies. This may be due to inappropriate sampling and cultural techniques, or because of selective pressures acting within the mouth which may prevent the colonization of bacteria despite their ability to adhere well. In addition, bacteria may be able to colonize teeth without adhering to enamel, but by becoming mechanically entrapped in sites such as the gingival crevice, or by forming complexes within a dental plaque matrix. Therefore, adherence cannot necessarily predict the *in vivo* colonizing potential of a bacterium on the tooth surface, although a high affinity *in vitro* may be a significant factor in colonization.

The results therefore suggest that adherence to the tooth surface may be important for most of the species tested, with the possible exceptions of H. aphrophilus, A. israelii and S. salivarius.

Some bacteria adhered to saliva treated enamel, buccal cells and saliva treated HeLa cells in similar proportions, whereas others showed quite different affinities for these surfaces. The similarities within a particular strain may be due to the presence of receptors specific for salivary components adsorbed to the surfaces of these materials. Strains demonstrating quite different affinities for the various surfaces may possess receptors specific for sites found only on particular oral surfaces.

CHAPTER 5

AGGREGATION OF ORAL BACTERIA IN VITRO

5.1 INTRODUCTION

The adherence of bacteria to epithelial cells and enamel surfaces was studied in the previous chapters. Other important surfaces available for microbial colonization in the mouth consist of pre-existing bacterial accumulations on teeth, on mucosal surfaces and in the gingival crevice. These microbial accumulations consist of many different bacterial species, which may possess distinct surface structures and so present an extremely variable surface available for colonization. Aggregation may also be necessary for the structural integrity of the plaque matrix and may therefore influence the subsequent development of bacterial plaques (McIntire, 1985).

The association between bacterial aggregation and dental disease has attracted much interest and consequently a number of methods have been developed to study aggregation phenomena. These methods vary from simple to complex techniques, but generally, the more rapid, simple methods provide only a limited amount of information (Bourgeau and McBride 1976). The most accurate method described previously appears to be the spectrophotometric method of Ericson, Pruitt and Wedel (1975), which was adapted for use in this study. With this technique, changes in optical density of an aggregating suspension of bacteria are monitored for a defined period and the data can be analysed in detail.

A number of the bacteria studied in the previous chapters failed to adhere well to any of the surfaces tested, and therefore, aggregation may be particularly important in enabling these bacteria to colonize the mouth. Homotypic aggregation of each of the 18 test bacteria was investigated in saliva, saliva ions buffer (SIB) and phosphate buffered saline (PBS). The information from these results was then used to select the most suitable medium in which to study heterotypic aggregation. All 18 isolates were tested for heterotypic aggregation with each other, giving 153 pairs of bacteria.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of bacterial suspensions

The bacteria detailed in Sections 2.2.1 to 2.2.5, were grown as described in Section 2.2.6. The cultures were then centrifuged at 3000g for 10 minutes in sterile disposable plastic universals (Nunc Inter Med, Kamstrup, Denmark) in an MSE super minor centrifuge (MSE Scientific Instruments, Crawley, England).

For the homotypic aggregation assay, the resultant pellet was resuspended in either saliva ions buffer (SIB) (Appendix 8) or phosphate buffered saline (PBS) (Appendix 9). The volumes of the suspensions were then adjusted with either SIB or PBS to an optical density of 1.5 (± 0.05) as determined with an SP 8-100 spectrophotometer (Pye Unicam Ltd., Cambridge, England) at a wavelength of 520 nm. The suspensions were diluted further with saliva or buffer to start the assay, giving a final optical density of approximately 1.2.

For the heterotypic aggregation assay, bacterial pellets were resuspended in PBS and the volumes were adjusted to give optical densities of 1.2 (± 0.05). No further dilutions were made prior to the assay, so the starting optical densities were similar to those used in the homotypic aggregation assay.

5.2.2 Determination of the optimum wavelength

The wavelength of light used for bacterial turbidity measurements was chosen after considering the absorption spectra of

anaerobe blood broth (ABB), SIB, PBS, clarified saliva and suspensions of bacteria in buffer. The absorption spectra of 3 ml volumes of the above were recorded between 350 nm and 750 nm in plastic cuvettes as used for the aggregation assays. A wavelength was then chosen that gave suitable optical density readings with the bacterial concentrations used, whilst the suspending media together with any contaminating broth gave minimum optical densities.

5.2.3 Homotypic aggregation assay

The method employed for measuring aggregation was based on the spectrophotometric method developed by Ericson et al. (1975). This assay quantifies aggregation of a bacterial suspension by monitoring any change in the absorbance of a suspension over a given period. If aggregation occurs, the initial formation of small non-sedimenting aggregates will result in a gradual decrease in the absorbance of the suspension. This may be followed by a more rapid decrease as larger aggregates form and begin to settle out. This process is illustrated in Figures 5.1 to 5.3 with heterotypically aggregating suspensions.

Homotypic aggregation assays were carried out in four different suspending media; SIB, PBS, saliva diluted in SIB, or saliva diluted in PBS. Whole mixed unstimulated saliva was collected and clarified as described in Section 3.2.4., with one exception; saliva used for the PBS plus saliva test was diluted with PBS prior to clarification. Saliva was stored at 4°C until use, and was always used on the day of collection. The saliva was diluted 1 in 2 prior to clarification, and then 1 in 3 in the aggregation assay, giving a final dilution of saliva of 1 in 6.

Figure 5.1 Top: cuvette containing a non-aggregating mixed suspension of S. salivarius P2 and Peptostreptococcus P2 after 2 hours incubation.

Bottom: Gram stain of the suspension at a magnification X 200.

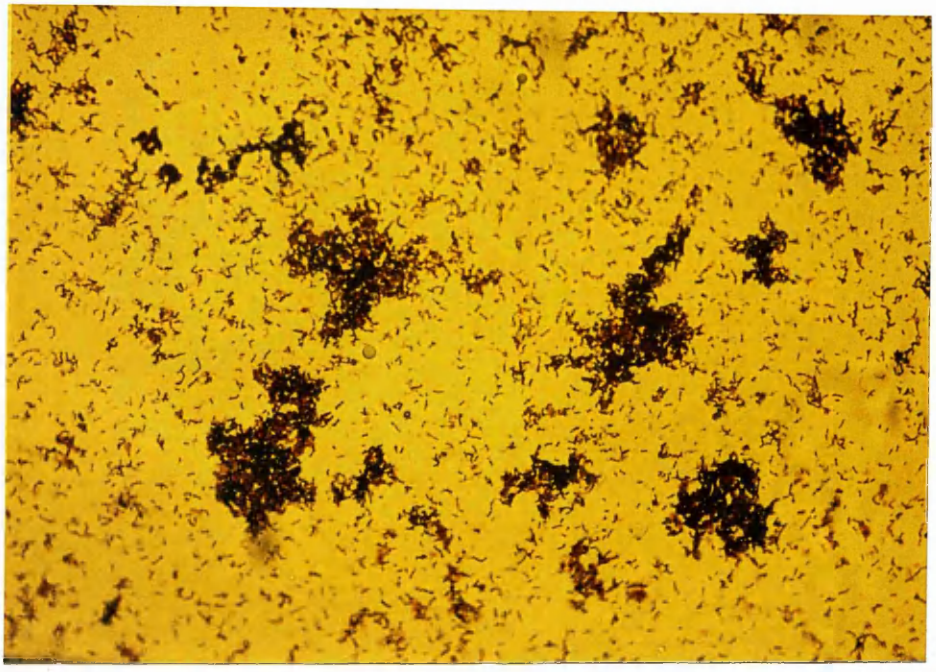
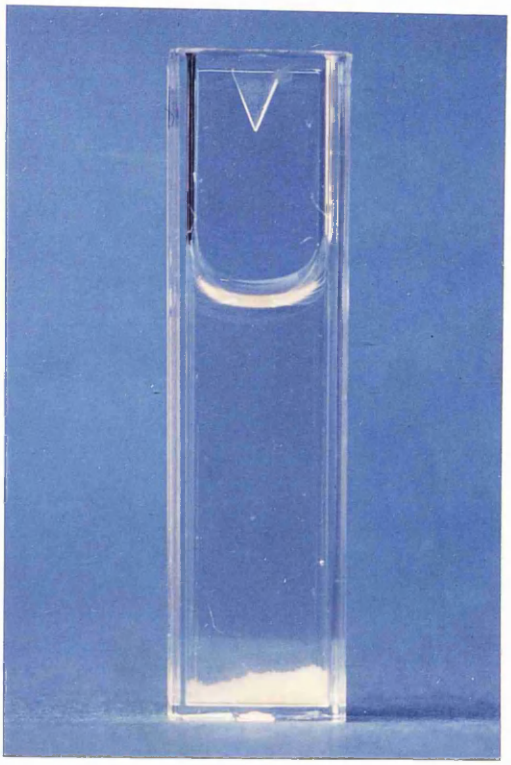


Figure 5.2 Top: a mixed suspension of S. salivarius P2 and Peptostreptococcus P2 after 2 hours incubation demonstrating visible bacterial aggregates.

Bottom: Gram stain of the suspension at a magnification X 200.



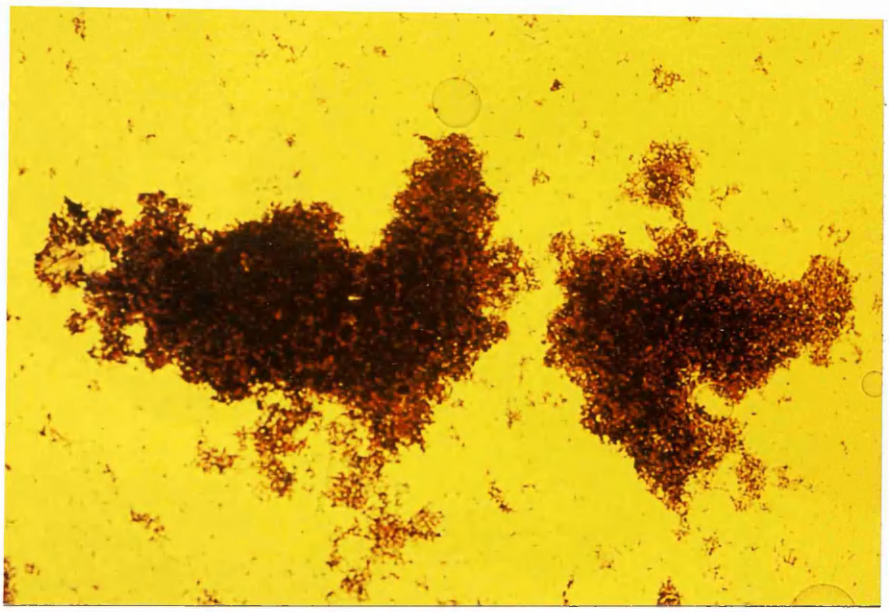


Figure 5.3 Top: a mixed suspensions of Peptostreptococcus P2 and B. intermedius P2 after 2 hours incubation showing strong aggregation of the bacteria and settling of the aggregates.

Bottom: Gram stain of the deposit at a magnification X 200.

The assays were performed with mixtures of bacterial suspensions and buffer or saliva in the following proportions:

2 ml bacterial suspension in SIB + 1 ml SIB

2 ml bacterial suspension in SIB + 1 ml saliva in SIB

2 ml bacterial suspension in PBS + 1 ml PBS

2 ml bacterial suspension in PBS + 1 ml saliva in PBS

The 3 ml volumes were mixed in plastic disposable cuvettes (Sarstedt Ltd., Leicester, England), with a path length of 10 mm, following the addition of the bacterial suspension to the saliva or buffer already in the cuvette. The cuvettes were sealed with Parafilm sealing tissue (A. Gallenkamp & Co. Ltd., London, England), inverted five times to thoroughly mix the contents, and then placed in the SP 8-100 spectrophotometer.

The measuring chamber of the spectrophotometer was kept at 37°C and the absorbance was monitored at 520 nm with a bandwidth of 1 nm. The measuring chamber accommodated four cuvettes which were automatically read in turn at predetermined intervals for a specified period. Typically, assays were carried out over a two hour period with readings taken every ten minutes. However, where aggregation was pronounced, the intervals between readings and the duration of the assays were reduced accordingly, for instance, with S. sanguis P1 in SIB, readings were taken every two minutes for thirty minutes. The spectrophotometer was linked to a Hewlett-Packard 97S calculator (Hewlett-Packard, Wokingham, Berkshire, England) which automatically printed out the absorbancies of the four cuvettes over the test period. Each experiment was performed on at least three occasions.

5.2.4 Heterotypic aggregation assay

The method used to measure heterotypic aggregation utilized the same principles, and was similar to that used to assess homotypic aggregation. The assays consisted of mixtures of 1.5 ml volumes of two different bacterial suspensions in PBS. All 18 strains of bacteria were tested with each other, giving 153 combinations. Bacterial suspensions were added to cuvettes and mixed by sealing with Parafilm and inverting five times. The cuvettes were then incubated in the spectrophotometer and monitored as in the homotypic aggregation assay. Controls consisted of 3 ml volumes of a single bacterium. To assess the amount of heterotypic aggregation occurring in each case, the results were compared with the controls representing the two bacteria in each combination. Each experiment was performed on at least three occasions.

The reactions were all performed in PBS, which was shown to induce the least amount of homotypic aggregation with the bacteria used. This was deemed necessary because if pronounced homotypic aggregation occurs during a heterotypic aggregation assay it may not be possible to distinguish the drop in absorbance caused by heterotypic aggregation from that caused by homotypic aggregation.

5.2.5 Calculation of aggregating activity

The data obtained from each aggregation assay gave a series of pairs of time and absorbance values. Graphs of absorbance versus time constructed from this data were found to give a sigmoidal curve, as shown in Figure 5.4. This curve demonstrates a typical plot, showing

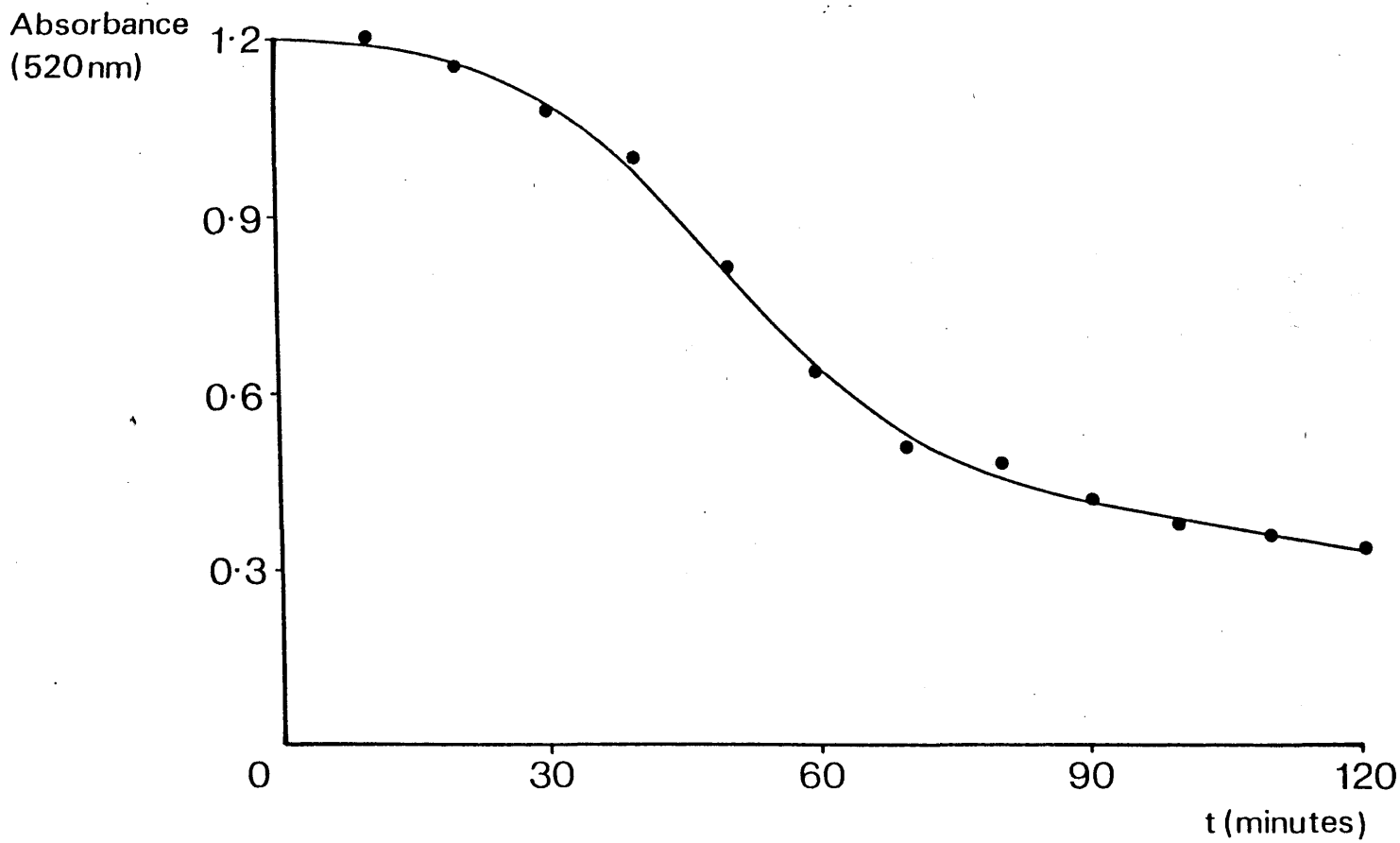


Figure 5.4 A typical curve showing the decrease in absorbance due to aggregation of a suspension of bacteria.

a decrease in absorbance of a bacterial suspension due to aggregation over the test period. The nature of this sigmoidal curve makes the analysis of the data more complicated than if the relationship between the two variables was linear. However, Ericson et al. (1975) developed an equation for the analysis of this kind of data (equation 1). This equation can be used to convert such data with a sigmoidal nature into a linear form and was used for data analyses in this study.

$$\ln\left[\frac{Ao - A}{A}\right] = mt + b \qquad \text{(equation 1)}$$

Where ln = natural logarithm

Ao = original absorbance

A = absorbance at time t

m = slope of a plot of ln((Ao-A)/A) versus time

b = intercept of a plot of ln((Ao-A)/A) versus time

By calculating graphs of ln((Ao-A)/A) versus time, linear functions can be derived, from which experimental parameters can be calculated to quantify each aggregation reaction. The curve illustrated in Figure 5.4 is a plot of absorbance versus time showing the typical sigmoidal nature of the curve as aggregation proceeds. Figure 5.5 shows the same data as that shown in Figure 5.4 converted to the form of ln((A-Ao)/A) versus time. It can be seen, however, that only a portion of the graph is in fact linear. Equation 1 was shown to hold true only for experimental data between approximately 0.95 and 0.60 of the original absorbance. Values outside of the range of 0.95 Ao to 0.60 Ao must be discounted, as in Figure 5.5, where only

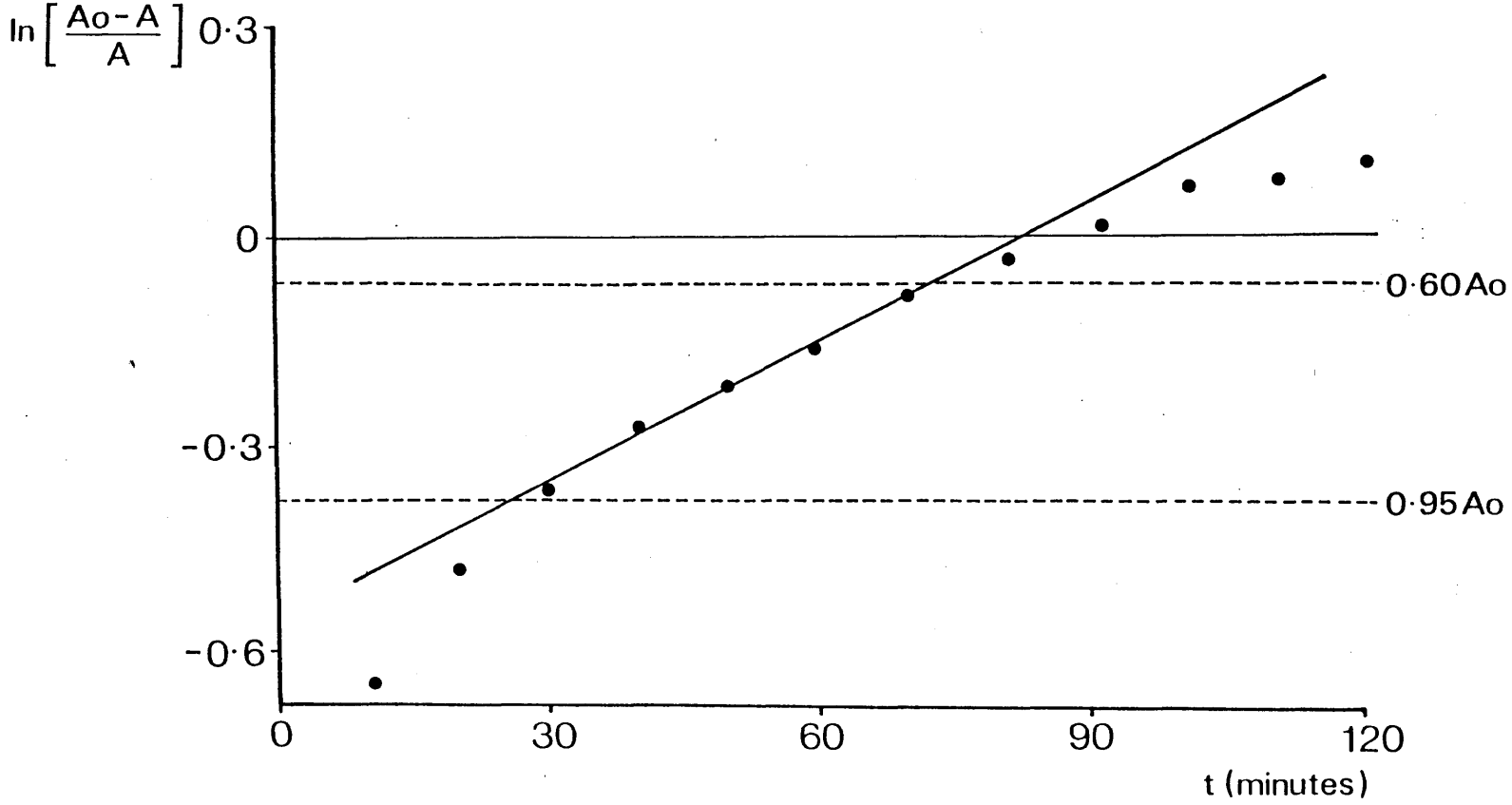


Figure 5.5 Data from figure 5.4 converted to the form derived from equation 1. N.B. only the points between 0.60 and 0.95 Ao (30 to 70 minutes) were used to construct the intersecting line.

the data between approximately 25 and 72 minutes can be used to construct the intersecting line.

Each aggregation reaction studied can be illustrated graphically. However, with large numbers of experiments, it becomes extremely difficult to study many sets of data in this way. Also the construction of the number of graphs required, would be extremely time consuming. Therefore, each set of aggregation data was represented by the original absorbance and two parameters calculated directly from numerical data using equation 1. The parameters used were:

(i) $t_{(Ao/2)}$ - the time required for the absorbance of a suspension to reach half of the original absorbance.

(ii) m - from equation 1, the slope of a plot of $\ln((Ao-A)/A)$ versus time, equivalent to the mean rate of aggregation.

Where a suspension demonstrates significant aggregating activity and the absorbance falls rapidly, $t_{(Ao/2)}$ will be small, for example, between 30 and 120 minutes. If no aggregation occurs, then any decrease in absorbance will be slight, due to simple sedimentation of single cells or possibly the lysis of some of the bacteria. Thus $t_{(Ao/2)}$ will be large, for example 600 minutes. In such cases, results were calculated by extrapolating from the data obtained over a two hour period. Values of m demonstrate an inverse relationship to $t_{(Ao/2)}$, so that the m value corresponding to a non-aggregating suspension will typically be less than 0.015. Aggregating suspensions will give larger values of m, between 0.025 and 0.300.

The original absorbancies will be similar regardless of the

aggregating system, since they are standardized for each test to the same approximate reading. The m values gives an indication of the form a particular aggregation reaction will follow. For example, in Figure 5.6 both curves have the same $t_{(Ao/2)}$ value (calculated from an extrapolated plot of ln((Ao-A)/A) versus time), but curve 1 has a larger m value than curve 2. This is due to a longer lag time by curve 1 before the absorbance decreased appreciably, compensated for by a more rapid drop in absorbance once initiated. Thus, the slope of curve 1, which determines the value of m, is twice that of curve 2, although the $t_{(Ao/2)}$ values are the same. Therefore the longer the lag phase, the larger the value of m for any particular $t_{(Ao/2)}$ value. Although m values are related to the rate of aggregation, the $t_{(Ao/2)}$ values were considered to be the best single descriptive parameter to represent the extent of aggregation. Therefore, $t_{(Ao/2)}$ values were primarily used for comparisons of the different tests.

The lag phase, which may precede the decrease in absorbance will influence the relationship between $t_{(Ao/2)}$ and m. To investigate this relationship for different experiments with large differences in m and $t_{(Ao/2)}$ values, the ratios between m and $t_{(Ao/2)}$ were calculated by multiplying these values; with regard to their inverse relationship.

The parameters $t_{(Ao/2)}$ and m can be derived from the graph shown in Figure 5.5. The $t_{(Ao/2)}$ occurs where the straight line plot crosses the x axis, ie. when ln((Ao-A)/A) equals zero; in the example shown, at 82 minutes. The parameter m is determined by measuring the slope of the line. The derivation of these values, however, was considerably simplified by calculating them directly from the numerical data, for which a computer programme was devised.

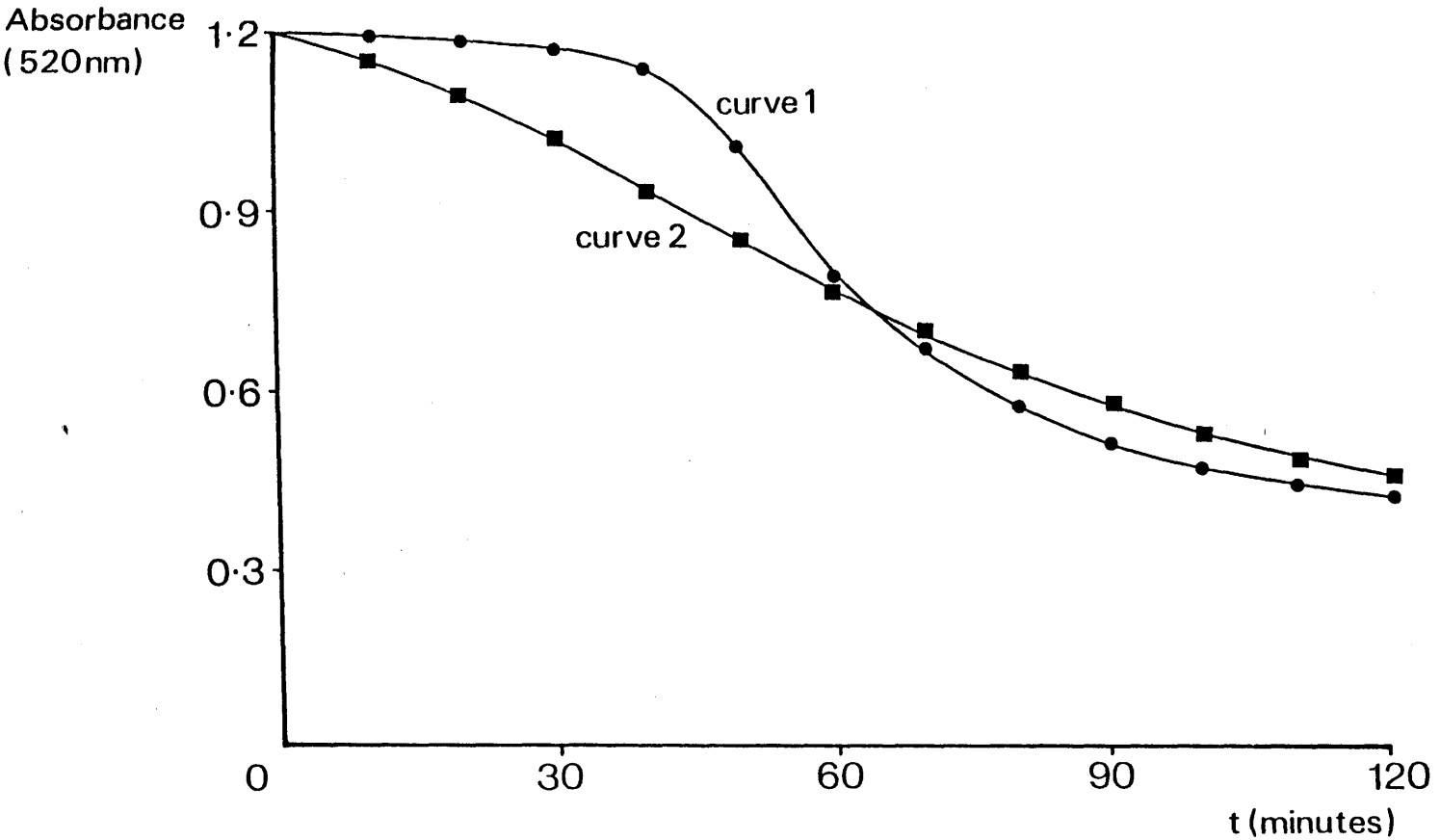


Figure 5.6 A comparison of two types of aggregation result.

Curve 1 - t(Ao/2) = 70 minutes; slope (m) = 0.10

Curve 2 - t(Ao/2) = 70 minutes; slope (m) = 0.05

5.2.6 Computation of $t_{(Ao/2)}$ and m

The parameters $t_{(Ao/2)}$ and m were calculated mathematically using a computer programme written in Applesoft basic for use on an Apple II computer (Apple Computers Inc., Cupertino, C.A., U.S.A).

The initial step of the programme calculates the experimental values within the range of 0.95 to 0.60 Ao that can be used to calculate the $t_{(Ao/2)}$ and m values. The original absorbance (Ao) is entered into the computer which responds with the range of acceptable values. The number of values lying within this range is then entered. The computer then requests the first pair of time and absorbance values and responds with the ln((Ao-A)/A) value for that pair of data. The remaining time and absorbance values are then entered in turn.

Calculation of m:

When all of the data has been assimilated, the programme applies a standard least squares regression analysis formula to calculate m:

$$m = \frac{\sum XY - \frac{(\sum X)(\sum Y)}{n}}{\sum X^2 - \frac{(\sum X)^2}{n}}$$

Let X = t, Y = ln((Ao-A)/A) and n = number of pairs of data.

$$m = \frac{\sum(t(\ln((Ao-A)/A))) - \frac{(\sum t)\,(\sum \ln((Ao-A)/A))}{n}}{\sum t^2 - \frac{(\sum t)^2}{n}} \qquad \text{(equation 2)}$$

Calculation of b:

Equation 1 states $\ln((Ao-A)/A) = mt + b$

Therefore $b = \ln((Ao-A)/A) - mt$

Entering the mean values for $\ln((Ao-A)/A)$ and t:

$$b = \frac{\sum \ln((Ao-A)/A)}{n} - \frac{m \sum t}{n}$$

$$b = \frac{\sum \ln((Ao-A)/A) - m \sum t}{n}$$

Calculation of $t_{(Ao/2)}$:

$$\text{At } t_{(Ao/2)} \qquad A = \frac{Ao}{2}$$

so $$\ln\left[\frac{Ao - A}{A}\right] = \ln(1) = 0$$

Therefore, from equation 1 $0 = mt_{(Ao/2)} + b$

$$t_{(Ao/2)} = \frac{-b}{m}$$

$$t_{(Ao/2)} = \frac{m \sum t - \sum \ln((Ao-A)/A)}{nm} \qquad \text{(equation 3)}$$

The computer calculates all of these values and gives a print-out of Ao, $t_{(Ao/2)}$ and m.

In addition, the computer was programmed to draw graphs of absorbance versus time and ln((Ao-A)/A) versus time. This allowed the immediate viewing of the course of the aggregation reaction in graphical form so that any experimental artifacts or operator errors could be seen that might otherwise have been missed.

5.2.7 Computation of correlation coefficients

The accurate determination of $t_{(Ao/2)}$ and m are dependent on the aggregation data within the specified range (0.90 to 0.65 Ao) fitting the relationship given in equation 1. If the aggregation data adequately fits this relationship then a plot of ln((Ao-A)/A) versus time will give a reasonably straight line. The more the data deviates from a linear relationship, the less likely is the data to be significant.

Points forming an exact straight line will give a coefficient of correlation (r) value of + or - 1, depending on whether the slope of the curve (m) is positive or negative, respectively. In the case of the aggregation data, the values of m and r are always positive as the values of ln((Ao-A)/A) always increase with time as the absorbance falls. As the data deviates from a linear correlation, r tends towards zero, and completely randomly distributed data will give a value of 0.

After calculating $t_{(Ao/2)}$ and m, the computer uses the same data and a standard statistical formula to calculate the coefficient of correlation and to predict at which level of probability r is significant for the relevant degrees of freedom. The r value and

level of significance are then printed out.

The values of r were generally between 0.95 and 0.99, corresponding to a significant correlation. Rarely, r was not significant due to an error in the recording or entering of results, or due to technical faults that occurred during the experiments. In such cases the data was checked and if necessary the experiment was repeated on another occasion.

5.2.8 Heterotypic aggregation controls

To determine if heterotypic aggregation has occurred in any particular aggregation mixture, the $t_{(Ao/2)}$ and m values should ideally be compared to those obtained from a non-aggregating suspension of the same two bacteria. However, it is not practically feasible to totally inhibit aggregation of such a mixture to provide an accurate control. Instead the test results were compared to two control suspensions of each bacterium incubated alone. To compare one test with two controls, it is necessary to calculate for each paired combination of controls the theoretical $t_{(Ao/2)}$ and m values if no aggregates are formed. This calculation is based on the assumption that a mixture of two bacteria which do not aggregate will give a drop in absorbance equal to the mean of that resulting in two separate control suspensions of the same bacteria.

To calculate the theoretical values of $t_{(Ao/2)}$ and m, the summation of two forms of equation 1 with two sets of variables were considered:

$$\ln\left[\frac{Ao - A}{A}\right] = m_1 t + b_1$$

$$\ln\left[\frac{Bo - B}{B}\right] = m_2 t + b_2$$

Let $t_{(Ao/2)} = T_1$

At T_1 $$A = \frac{Ao}{2}$$

Hence $$\ln\left[\frac{Ao - A}{A}\right] = \ln(1) = 0$$

Therefore $$0 = m_1 T_1 + b_1$$

$$b_1 = - m_1 T_1$$

Therefore $$\ln\left[\frac{Ao - A}{A}\right] = m_1 t - m_1 T_1$$

Let the time at which the absorbance of the combined control suspensions reaches half the original absorbance equal t_x, when $A = A_x$ and $B = B_x$.

Therefore, at t_x

$$\ln\left[\frac{Ao - A_x}{A_x}\right] = m_1 t_x - m_1 T_1$$

$$\frac{Ao - A_x}{A_x} = \ln^{-1}(m_1 t_x - m_1 T_1)$$

$$Ao = A_x(\ln^{-1}(m_1 t_x - m_1 T_1)) + A_x$$

$$Ao = A_x(1 + \ln^{-1}(m_1 t_x - m_1 T_1))$$

hence $$A_x = \frac{Ao}{1 + \ln^{-1}(m_1 t_x - m_1 T_1)}$$

similarly $$B_x = \frac{Bo}{1 + \ln^{-1}(m_2 t_x - m_2 T_2)}$$

Therefore

$$A_x + B_x = \frac{Ao}{1 + \ln^{-1}(m_1 t_x - m_1 T_1)} + \frac{Bo}{1 + \ln^{-1}(m_2 t_x - m_2 T_2)}$$

At t_x $$A_x + B_x = \frac{Ao + Bo}{2}$$

Therefore

$$\frac{Ao}{1 + \ln^{-1}(m_1 t_x - m_1 T_1)} + \frac{Bo}{1 + \ln^{-1}(m_2 t_x - m_2 T_2)} = \frac{Ao + Bo}{2}$$

A computer programme was written to determine the value of t_x for which this equation holds true, which is the predicted $t_{(Ao/2)}$ for a non-aggregating mixture of two control suspensions. The computer programme was used to calculate the 153 possible combinations of control results which corresponded to the 153 test results.

5.2.9 Statistical analyses

Each aggregation assay was performed on at least three occasions on different days. The means of the original absorbancies, m and $t_{(Ao/2)}$ values were determined and the standard errors of the means were calculated using standard statistical formulae. The significance of differences between results were determined using the Mann-Whitney U test. Correlation coefficients were calculated for comparisons of the aggregation results with the adherence results from the previous chapters.

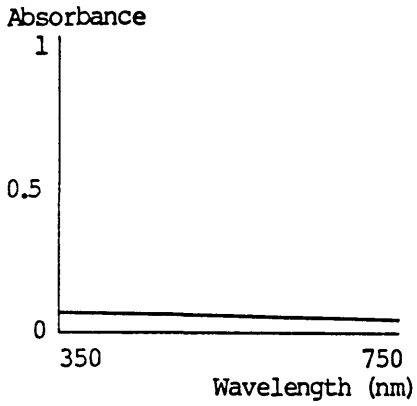
5.3 RESULTS

5.3.1 Determination of the optimum wavelength

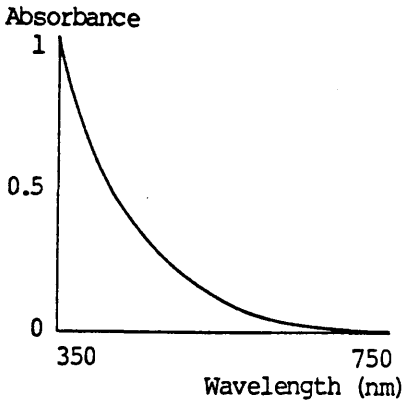
Graphical representations of the absorbance spectra of anaerobe blood broth (ABB), SIB, PBS, clarified saliva and a suspension of bacteria in buffer are shown in Figure 5.7. The absorption spectra of the two buffers and saliva show a negligible absorbance over the whole range, falling only slightly as the wavelength increases. The absorbancies of the ABB and the bacterial suspension in buffer both decrease with increasing absorbance up to 750 nm. However, the absorbance of the bacterial suspension decreases linearly while that of the ABB decreases approximately exponentially.

The optimum wavelength for the assay was taken as the point that gave sufficiently large absorbance values with the concentrations of bacteria used, but gave low absorbancies with the suspending media used and any contaminating ABB carried over into the suspensions. Since the absorbancies of the suspending media were negligible, this point was found by combining the two graphs of the ABB and the bacterial suspension spectra as shown in Figure 5.7. The point at which the difference between the two plots was maximal gave absorbance values within the range required and was therefore taken as the optimal wavelength, which occurred at 520 nm.

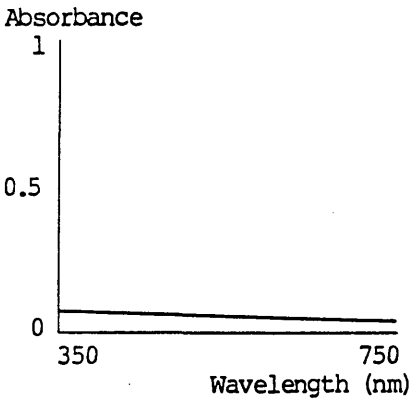
i. Saliva ions buffer.



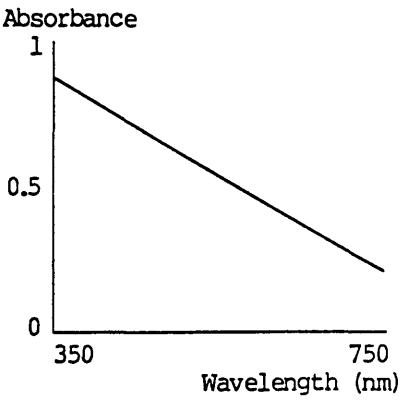
iv. Anaerobe blood broth.



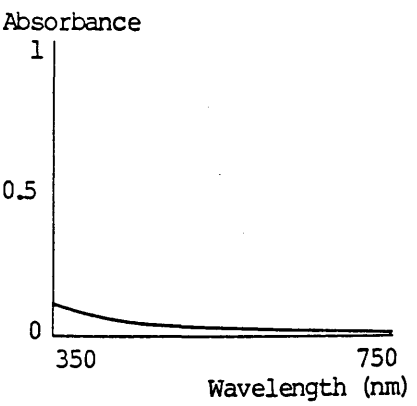
ii. Phosphate buffered saline.



v. Bacterial suspension.



iii. Saliva



vi. Curves iv and v superimposed.

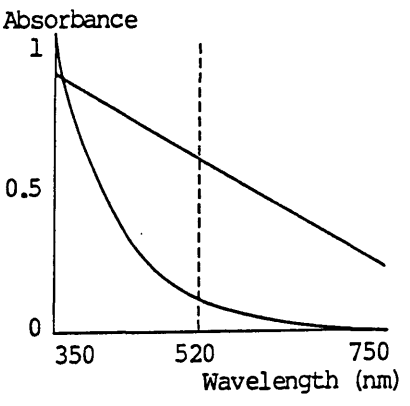


Figure 5.7 Graphical representations of the absorption spectra taken into consideration to determine the optimum wavelength (520 nm) used in the aggregation assays.

5.3.2 Homotypic aggregation results

Homotypic aggregation was tested in SIB, SIB plus saliva, PBS and PBS plus saliva. The results are listed in Tables 5.1 to 5.4, respectively, the $t_{(Ao/2)}$ values are summarized in Table 5.5. The $t_{(Ao/2)}$ values ranged from 21 minutes, representing rapid aggregation, to 1502 minutes where the decrease in absorbance was due to simple sedimentation of unaggregated bacteria. The values of m ranged from 0.222 to 0.002, representing rapid aggregation and simple sedimentation, respectively. The original absorbancies (Ao) were relatively consistent as they were standardized for each experiment.

The ratios between m and $t_{(Ao/2)}$ were calculated by multiplying these values, and are listed in Table 5.6. The amount of variance noted between the ratios of the different experiments is small considering the large differences in the m and $t_{(Ao/2)}$ values listed in Tables 5.1 to 5.4. There was no significant difference between the means of the ratios of any of the suspending media used, nor of the aggregating suspensions compared with non-aggregating suspensions. This infers that the patterns of decrease of absorbance are similar for all of the bacteria tested in any of the suspending media, regardless of whether aggregation was pronounced or negligible.

To compare the different results, it is preferable to consider just one variable. For this purpose, the $t_{(Ao/2)}$ results, as compared to the m values, were considered to give the clearest indication of the amount of aggregation. Marked aggregation was considered subjectively as a suspension giving a $t_{(Ao/2)}$ of less than 100 minutes, although the results obviously cannot be divided precisely

Table 5.1 Homotypic aggregation in SIB.

Bacterium	Ao	m	$t_{(Ao/2)}$* ± SEM
B. gingivalis P4	1.133	0.017	166 ± 16
B. gingivalis W83	1.126	0.006	636 ± 30
B. intermedius P2	1.138	0.013	249 ± 12
B. intermedius 9336	1.152	0.008	493 ± 58
Capnocytophaga sp. P2	1.143	0.006	540 ± 46
Capnocytophaga sp. 27872	1.065	0.010	349 ± 62
H. aphropilus P5	1.107	0.166	24 ± 0
H. actinomycetemc. 9710	1.093	0.049	61 ± 2
Peptostrep. sp. P2	1.126	0.007	345 ± 12
Peptostrep. sp. 9807	1.189	0.008	472 ± 81
Veillonella sp. P3	1.125	0.222	22 ± 2
Veillonella sp. 11463	1.001	0.016	214 ± 31
A. israelii P2	1.053	0.012	281 ± 43
A. israelii 10215	1.125	0.088	50 ± 9
S. salivarius P2	1.183	0.107	43 ± 11
S. salivarius 8618	1.200	0.007	453 ± 61
S. sanguis P1	1.088	0.144	21 ± 1
S. sanguis 7863	1.211	0.009	405 ± 51

*$t_{(Ao/2)}$ in minutes.

Table 5.2 Homotypic aggregation in SIB plus saliva.

Bacterium	Ao	m	$t_{(Ao/2)}$* ± SEM
B. gingivalis P4	1.175	0.012	292 ± 48
B. gingivalis W83	1.147	0.004	878 ± 69
B. intermedius P2	1.140	0.015	184 ± 8
B. intermedius 9336	1.170	0.006	626 ± 75
Capnocytophaga sp. P2	1.172	0.117	32 ± 3
Capnocytophaga sp. 27872	1.057	0.029	78 ± 1
H. aphrophilus P5	1.114	0.112	32 ± 2
H. actinomycetemc. 9710	1.090	0.024	111 ± 22
Peptostrep. sp. P2	1.094	0.058	56 ± 4
Peptostrep. sp. 9807	1.152	0.026	212 ± 34
Veillonella sp. P3	1.132	0.055	56 ± 4
Veillonella sp. 11463	1.011	0.066	36 ± 3
A. israelii P2	1.077	0.013	257 ± 30
A. israelii 10215	1.138	0.070	54 ± 16
S. salivarius P2	1.202	0.115	39 ± 5
S. salivarius 8618	1.195	0.156	39 ± 3
S. sanguis P1	1.096	0.112	25 ± 2
S. sanguis 7863	1.228	0.068	61 ± 12

*$t_{(Ao/2)}$ in minutes.

Table 5.3 Homotypic aggregation in PBS.

Bacterium	Ao	m	$t_{(Ao/2)}$* ± SEM
B. gingivalis P4	1.166	0.014	371 ± 42
B. gingivalis W83	1.106	0.005	616 ± 66
B. intermedius P2	1.136	0.019	163 ± 9
B. intermedius 9336	1.148	0.012	340 ± 12
Capnocytophaga sp. P2	1.130	0.012	239 ± 2
Capnocytophaga sp. 27872	1.092	0.008	369 ± 27
H. aphrophilus P5	1.073	0.025	108 ± 21
H. actinomycetemc. 9710	1.085	0.007	355 ± 20
Peptostrep. sp. P2	1.129	0.008	336 ± 3
Peptostrep. sp. 9807	1.214	0.007	635 ± 91
Veillonella sp. P3	1.159	0.023	135 ± 17
Veillonella sp. 11463	1.072	0.009	367 ± 50
A. israelii P2	1.038	0.010	325 ± 42
A. israelii 10215	1.136	0.019	146 ± 5
S. salivarius P2	1.211	0.012	273 ± 5
S. salivarius 8618	1.200	0.009	395 ± 20
S. sanguis P1	1.021	0.009	260 ± 17
S. sanguis 7863	1.208	0.009	328 ± 26

*$t_{(Ao/2)}$ in minutes.

Table 5.4 Homotypic aggregation in PBS plus saliva.

Bacterium	Ao	m	$t_{(Ao/2)}$* ± SEM
B. gingivalis P4	1.196	0.013	246 ± 9
B. gingivalis W83	1.121	0.002	1502 ±147
B. intermedius P2	1.135	0.023	126 ± 11
B. intermedius 9336	1.165	0.007	606 ± 98
Capnocytophaga sp. P2	1.153	0.052	65 ± 10
Capnocytophaga sp. 27872	1.090	0.021	97 ± 18
H. aphrophilus P5	1.051	0.083	45 ± 3
H. actinomycetemc. 9710	1.088	0.003	931 ± 35
Peptostrep. sp. P2	1.128	0.064	48 ± 4
Peptostrep. sp. 9807	1.215	0.009	354 ± 50
Veillonella sp. P3	1.172	0.021	145 ± 6
Veillonella sp. 11463	1.023	0.106	26 ± 3
A. israelii P2	1.065	0.012	274 ± 50
A. israelii 10215	1.160	0.043	104 ± 24
S. salivarius P2	1.215	0.048	88 ± 21
S. salivarius 8618	1.216	0.159	35 ± 2
S. sanguis P1	1.060	0.109	23 ± 2
S. sanguis 7863	1.226	0.057	73 ± 14

*$t_{(Ao/2)}$ in minutes.

Table 5.5 Homotypic aggregation $t_{(Ao/2)}$* results summary for SIB, PBS, SIB plus saliva and PBS plus saliva.

Bacterium	SIB	SIB plus saliva	PBS	PBS plus saliva
B. gingivalis P4	166	292	371	246+
B. gingivalis W83	636	878	616	1502
B. intermedius P2	249	184	163	126+
B. intermedius 9336	493	626	340	606
Capnocytophaga sp. P2	540	32	239	65+
Capnocytophaga sp. 27872	549	78	369	97+
H. aphrophilus P5	24	32	108	45+
H. actinomycetemc. 9710	61	111	355	931
Peptostrep. sp. P2	345	56	336	48+
Peptostrep. sp. 9807	472	212	635	354+
Veillonella sp. P3	22	56	135	145
Veillonella sp. 11463	214	36	367	26+
A. israelii P2	281	257	325	274
A. israelii 10215	50	54	146	104
S. salivarius P2	43	39	273	88+
S. salivarius 8618	453	39	395	35+
S. sanguis P1	21	25	260	23+
S. sanguis 7863	405	61	328	73+

*$t_{(Ao/2)}$ in minutes.

+Significantly lower $t_{(Ao/2)}$ than with PBS alone ($p < 0.05$).

Table 5.6 Ratios between m and $t_{(Ao/2)}$ for SIB, PBS, SIB plus saliva and PBS plus saliva.

Bacterium	SIB	SIB plus saliva	PBS	PBS plus saliva
B. gingivalis P4	2.8	3.5	5.2	3.2
B. gingivalis W83	3.8	3.5	3.1	3.0
B. intermedius P2	3.2	2.8	3.1	2.9
B. intermedius 9336	3.9	3.8	4.1	4.2
Capnocytophaga sp. P2	3.2	3.7	2.9	3.4
Capnocytophaga sp. 27872	3.5	2.3	3.0	2.0
H. aphrophilus P5	4.0	3.6	2.7	3.7
H. actinomycetemc. 9710	3.0	2.7	2.5	2.8
Peptostrep. sp. P2	2.4	3.2	2.7	3.1
Peptostrep. sp. 9807	3.8	5.5	4.4	3.2
Veillonella sp. P3	4.9	3.1	3.1	3.0
Veillonella sp. 11463	3.4	2.4	3.3	2.8
A. israelii P2	3.4	3.3	3.3	3.3
A. israelii 10215	4.4	3.8	2.8	4.5
S. salivarius P2	4.6	4.4	3.3	4.2
S. salivarius 8618	3.2	6.1	3.6	5.6
S. sanguis P1	3.0	2.8	2.3	2.5
S. sanguis 7863	3.6	4.1	3.0	4.2
MEAN	3.6	3.6	3.2	3.4
SEM	±0.15	±0.23	±0.17	±0.20

between aggregating and non-aggregating suspensions.

Large differences were evident in the amounts of bacterial aggregation induced by SIB and PBS (Table 5.5). None of the bacteria tested aggregated markedly in PBS, although six strains aggregated in SIB, namely H. aphrophilus P5, H. actinomycetemcomitans 9710, Veillonella species P3, A. israelii 10215, S. salivarius P2 and S. sanguis P1. Because PBS caused little aggregation of the bacteria tested, saliva induced aggregation was more clearly seen with saliva diluted in PBS. Bacteria that aggregated in PBS plus saliva also aggregated in SIB plus saliva, suggesting that the buffers used did not affect salivary aggregation.

A comparison of the PBS and PBS plus saliva results showed that twelve strains (marked ^{x} in Table 5.5) gave significantly lower $t_{(Ao/2)}$ values in PBS plus saliva ($p < 0.05$), suggesting that these strains are aggregated by saliva. Nine of these strains showed marked salivary aggregation with $t_{(Ao/2)}$ values of less than 100 minutes. B. gingivalis P4, B. intermedius P2 and Peptostreptococcus species 9807 aggregated only weakly with saliva, giving $t_{(Ao/2)}$ values of 246, 126 and 354 minutes respectively. In addition, SIB aggregated three bacteria that did not aggregate in saliva, namely H. actinomycetemcomitans 9710, Veillonella species P3 and A. israelii 10215.

With several strains, suspensions in saliva gave significantly larger $t_{(Ao/2)}$ values ($p < 0.05$) than buffer only suspensions. This effect appears to be due to the viscosity of saliva retarding the sedimentation of bacterial aggregates or non-aggregated single cells.

A comparison of the fresh and type strains showed that the fresh strains mainly gave significantly lower $t_{(Ao/2)}$ values in all four suspending media. The main exceptions were the A. israelii strains which produced the converse result in all four media, and the Veillonella and S. salivarius strains in the buffer plus saliva tests. The two latter exceptions resulted because the type strains aggregated in saliva although the fresh strains did not. However, overall the fresh and type strains gave similar results as regards salivary aggregation. Of the twelve strains that aggregated in saliva, seven were fresh strains and five were type strains. In addition, six strains aggregated in SIB, of which four were fresh strains and two were type strains.

5.3.3 Heterotypic aggregation results

The heterotypic aggregation results are listed in Table 5.7 and consists of 153 different combinations of bacteria in PBS. The $t_{(Ao/2)}$ values are summarized in Table 5.8. To assess the amount of heterotypic aggregation occurring in each case, the results were compared with the controls representing the two bacteria in each combination. The controls are essentially the same as the homotypic aggregation assays in PBS, but because of the slightly different assay procedure used for the heterotypic aggregation assay, controls were prepared specifically for this assay. The control results are listed in Table 5.9, and compare closely with the PBS homotypic aggregation assay results in Table 5.3. The results in Table 5.9 were used to calculate the 153 possible combinations of predicted non-aggregating control results (Table 5.10) corresponding to the 153 test results.

Table 5.7 Heterotypic aggregation in PBS.

Bacterial combination		Ao	m	$t_{(Ao/2)}$*
B. gingivalis P4	+ B. gingivalis W83	1.206	0.007	566 ± 48
B. gingivalis P4	+ B. intermedius P2	1.179	0.024	125 ± 10
B. gingivalis P4	+ B. intermedius 9336	1.167	0.012	239 ± 12
B. gingivalis P4	+ Capnocyt. sp. P2	1.138	0.007	482 ± 58
B. gingivalis P4	+ Capnocyt. sp. 27872	1.161	0.025	190 ± 19
B. gingivalis P4	+ H. aphrophilus P5	1.105	0.017	238 ± 15
B. gingivalis P4	+ H. actinomyc. 9710	1.164	0.007	491 ± 75
B. gingivalis P4	+ Peptostr. sp. P2	1.136	0.007	448 ± 69
B. gingivalis P4	+ Peptostr. sp. 9807	1.186	0.009	392 ± 8
B. gingivalis P4	+ Veillon. sp. P3	1.167	0.020	168 ± 6
B. gingivalis P4	+ Veillon. sp. 11463	1.182	0.008	457 ± 40
B. gingivalis P4	+ A. israelii P2	1.185	0.009	424 ± 43
B. gingivalis P4	+ A. israelii 10215	1.132	0.020	141 ± 9
B. gingivalis P4	+ S. salivarius P2	1.149	0.014	221 ± 18
B. gingivalis P4	+ S. salivarius 8618	1.210	0.011	285 ± 32
B. gingivalis P4	+ S. sanguis P1	1.172	0.013	195 ± 14
B. gingivalis P4	+ S. sanguis 7863	1.156	0.023	142 ± 13
B. gingivalis W83	+ B. intermedius P2	1.181	0.017	143 ± 3
B. gingivalis W83	+ B. intermedius 9336	1.187	0.009	299 ± 6
B. gingivalis W83	+ Capnocyt. sp. P2	1.174	0.010	331 ± 16
B. gingivalis W83	+ Capnocyt. sp. 27872	1.216	0.020	164 ± 15
B. gingivalis W83	+ H. aphrophilus P5	1.141	0.012	240 ± 23

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.7 (continued) Heterotypic aggregation in PBS.

Bacterial combination		Ao	m	$t_{(Ao/2)}$*
B. gingivalis W83	+ H. actinomyc. 9710	1.183	0.005	644 ± 64
B. gingivalis W83	+ Peptostr. sp. P2	1.178	0.008	366 ± 3
B. gingivalis W83	+ Peptostr. sp. 9807	1.176	0.005	600 ± 42
B. gingivalis W83	+ Veillon. sp. P3	1.171	0.015	177 ± 2
B. gingivalis W83	+ Veillon. sp. 11463	1.197	0.010	302 ± 2
B. gingivalis W83	+ A. israelii P2	1.199	0.011	337 ± 26
B. gingivalis W83	+ A. israelii 10215	1.132	0.029	104 ± 15
B. gingivalis W83	+ S. salivarius P2	1.184	0.010	306 ± 22
B. gingivalis W83	+ S. salivarius 8618	1.230	0.010	283 ± 20
B. gingivalis W83	+ S. sanguis P1	1.184	0.014	176 ± 5
B. gingivalis W83	+ S. sanguis 7863	1.183	0.019	158 ± 10
B. intermedius P2	+ B. intermedius 9336	1.169	0.015	215 ± 11
B. intermedius P2	+ Capnocyt. sp. P2	1.160	0.014	248 ± 19
B. intermedius P2	+ Capnocyt. sp. 27872	1.223	0.026	142 ± 5
B. intermedius P2	+ H. aphrophilus P5	1.161	0.060	54 ± 3
B. intermedius P2	+ H. actinomyc. 9710	1.126	0.017	151 ± 4
B. intermedius P2	+ Peptostr. sp. P2	1.139	0.055	60 ± 1
B. intermedius P2	+ Peptostr. sp. 9807	1.190	0.016	197 ± 8
B. intermedius P2	+ Veillon. sp. P3	1.176	0.034	96 ± 6
B. intermedius P2	+ Veillon. sp. 11463	1.185	0.021	161 ± 15
B. intermedius P2	+ A. israelii P2	1.171	0.022	146 ± 6
B. intermedius P2	+ A. israelii 10215	1.041	0.412	8 ± 2

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.7 (continued) Heterotypic aggregation in PBS.

Bacterial combination	Ao	m	$t_{(Ao/2)}$*
B. intermedius P2 + S. salivarius P2	1.208	0.036	93 ± 2
B. intermedius P2 + S. salivarius 8618	1.200	0.046	77 ± 6
B. intermedius P2 + S. sanguis P1	1.150	0.062	52 ± 4
B. intermedius P2 + S. sanguis 7863	1.164	0.038	96 ± 6
B. intermedius 9336 + Capnocyt. sp. P2	1.125	0.010	307 ± 19
B. intermedius 9336 + Capnocyt. sp. 27872	1.182	0.012	295 ± 20
B. intermedius 9336 + H. aphrophilus P5	1.119	0.017	178 ± 1
B. intermedius 9336 + H. actinomyc. 9710	1.136	0.009	356 ± 8
B. intermedius 9336 + Peptostr. sp. P2	1.162	0.008	382 ± 29
B. intermedius 9336 + Peptostr. sp. 9807	1.174	0.008	433 ± 38
B. intermedius 9336 + Veillon. sp. P3	1.161	0.021	162 ± 9
B. intermedius 9336 + Veillon. sp. 11463	1.173	0.010	348 ± 8
B. intermedius 9336 + A. israelii P2	1.165	0.012	355 ± 11
B. intermedius 9336 + A. israelii 10215	1.120	0.015	208 ± 21
B. intermedius 9336 + S. salivarius P2	1.196	0.010	343 ± 2
B. intermedius 9336 + S. salivarius 8618	1.236	0.012	281 ± 4
B. intermedius 9336 + S. sanguis P1	1.143	0.011	250 ± 18
B. intermedius 9336 + S. sanguis 7863	1.143	0.010	330 ± 7
Capnocyt. sp. P2 + Capnocyt. sp. 27872	1.135	0.008	380 ± 4
Capnocyt. sp. P2 + H. aphrophilus P5	1.111	0.012	217 ± 11
Capnocyt. sp. P2 + H. actinomyc. 9710	1.128	0.008	377 ± 1
Capnocyt. sp. P2 + Peptostr. sp. P2	1.183	0.009	392 ± 19

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.7 (continued) Heterotypic aggregation in PBS.

Bacterial combination		Ao	m	$t_{(Ao/2)}$*
Capnocyt. sp. P2	+ Peptostr. sp. 9807	1.188	0.006	565 ± 1
Capnocyt. sp. P2	+ Veillon. sp. P3	1.173	0.017	156 ± 2
Capnocyt. sp. P2	+ Veillon. sp. 11463	1.175	0.009	415 ± 18
Capnocyt. sp. P2	+ A. israelii P2	1.179	0.009	375 ± 3
Capnocyt. sp. P2	+ A. israelii 10215	1.137	0.013	195 ± 4
Capnocyt. sp. P2	+ S. salivarius P2	1.143	0.009	280 ± 1
Capnocyt. sp. P2	+ S. salivarius 8618	1.189	0.007	457 ± 11
Capnocyt. sp. P2	+ S. sanguis P1	1.162	0.009	306 ± 24
Capnocyt. sp. P2	+ S. sanguis 7863	1.185	0.010	337 ± 3
Capnocyt. sp. 27872	+ H. aphrophilus P5	1.146	0.017	182 ± 6
Capnocyt. sp. 27872	+ H. actinomyc. 9710	1.207	0.008	359 ± 26
Capnocyt. sp. 27872	+ Peptostr. sp. P2	1.190	0.009	391 ± 30
Capnocyt. sp. 27872	+ Peptostr. sp. 9807	1.216	0.009	416 ± 39
Capnocyt. sp. 27872	+ Veillon. sp. P3	1.212	0.018	168 ± 10
Capnocyt. sp. 27872	+ Veillon. sp. 11463	1.204	0.007	589 ± 53
Capnocyt. sp. 27872	+ A. israelii P2	1.176	0.016	259 ± 28
Capnocyt. sp. 27872	+ A. israelii 10215	1.162	0.033	89 ± 4
Capnocyt. sp. 27872	+ S. salivarius P2	1.250	0.012	271 ± 24
Capnocyt. sp. 27872	+ S. salivarius 8618	1.282	0.009	389 ± 8
Capnocyt. sp. 27872	+ S. sanguis P1	1.204	0.008	297 ± 10
Capnocyt. sp. 27872	+ S. sanguis 7863	1.238	0.010	346 ± 18
H. aphrophilus P5	+ H. actinomyc. 9710	1.059	0.008	348 ± 9

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.7 (continued) Heterotypic aggregation in PBS.

Bacterial combination		Ao	m	$t_{(Ao/2)}$*
H. aphrophilus P5	+ Peptostr. sp. P2	1.147	0.014	212 ± 27
H. aphrophilus P5	+ Peptostr. sp. 9807	1.141	0.011	257 ± 23
H. aphrophilus P5	+ Veillon. sp. P3	1.158	0.019	136 ± 5
H. aphrophilus P5	+ Veillon. sp. 11463	1.141	0.015	197 ± 3
H. aphrophilus P5	+ A. israelii P2	1.121	0.021	167 ± 16
H. aphrophilus P5	+ A. israelii 10215	1.126	0.087	33 ± 3
H. aphrophilus P5	+ S. salivarius P2	1.163	0.018	148 ± 10
H. aphrophilus P5	+ S. salivarius 8618	1.191	0.018	136 ± 9
H. aphrophilus P5	+ S. sanguis P1	1.177	0.043	64 ± 2
H. aphrophilus P5	+ S. sanguis 7863	1.130	0.013	221 ± 21
H. actinomyc. 9710	+ Peptostr. sp. P2	1.178	0.008	471 ± 4
H. actinomyc. 9710	+ Peptostr. sp. 9807	1.174	0.006	580 ± 34
H. actinomyc. 9710	+ Veillon. sp. P3	1.090	0.017	163 ± 8
H. actinomyc. 9710	+ Veillon. sp. 11463	1.099	0.011	306 ± 14
H. actinomyc. 9710	+ A. israelii P2	1.174	0.010	347 ± 17
H. actinomyc. 9710	+ A. israelii 10215	1.147	0.016	222 ± 22
H. actinomyc. 9710	+ S. salivarius P2	1.158	0.010	383 ± 11
H. actinomyc. 9710	+ S. salivarius 8618	1.215	0.009	306 ± 10
H. actinomyc. 9710	+ S. sanguis P1	1.150	0.011	241 ± 17
H. actinomyc. 9710	+ S. sanguis 7863	1.170	0.008	416 ± 13
Peptostr. sp. P2	+ Peptostr. sp. 9807	1.115	0.008	406 ± 17
Peptostr. sp. P2	+ Veillon. sp. P3	1.121	0.017	186 ± 9

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.7 (continued) Heterotypic aggregation in PBS.

Bacterial combination		Ao	m	$t_{(Ao/2)}$*
Peptostr. sp. P2	+ Veillon. sp. 11463	1.037	0.006	583 ± 52
Peptostr. sp. P2	+ A. israelii P2	1.144	0.009	368 ± 18
Peptostr. sp. P2	+ A. israelii 10215	1.093	0.013	179 ± 25
Peptostr. sp. P2	+ S. salivarius P2	1.132	0.010	342 ± 22
Peptostr. sp. P2	+ S. salivarius 8618	1.169	0.007	392 ± 14
Peptostr. sp. P2	+ S. sanguis P1	1.141	0.010	281 ± 6
Peptostr. sp. P2	+ S. sanguis 7863	1.194	0.008	333 ± 16
Peptostr. sp. 9807	+ Veillon. sp. P3	1.137	0.016	196 ± 6
Peptostr. sp. 9807	+ Veillon. sp. 11463	1.076	0.007	528 ± 29
Peptostr. sp. 9807	+ A. israelii P2	1.146	0.008	513 ± 64
Peptostr. sp. 9807	+ A. israelii 10215	1.130	0.010	311 ± 27
Peptostr. sp. 9807	+ S. salivarius P2	1.141	0.007	475 ± 45
Peptostr. sp. 9807	+ S. salivarius 8618	1.199	0.007	458 ± 39
Peptostr. sp. 9807	+ S. sanguis P1	1.149	0.009	347 ± 28
Peptostr. sp. 9807	+ S. sanguis 7863	1.053	0.007	436 ± 29
Veillon. sp. P3	+ Veillon. sp. 11463	1.122	0.023	167 ± 1
Veillon. sp. P3	+ A. israelii P2	1.157	0.021	186 ± 7
Veillon. sp. P3	+ A. israelii 10215	1.147	0.019	160 ± 13
Veillon. sp. P3	+ S. salivarius P2	1.149	0.041	63 ± 3
Veillon. sp. P3	+ S. salivarius 8618	1.171	0.102	37 ± 1
Veillon. sp. P3	+ S. sanguis P1	1.161	0.023	116 ± 21
Veillon. sp. P3	+ S. sanguis 7863	1.109	0.019	155 ± 3

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.7 (continued) Heterotypic aggregation in PBS.

Bacterial combination		Ao	m	$t_{(Ao/2)}$*
Veillon. sp. 11463	+ A. israelii P2	1.126	0.009	410 ± 34
Veillon. sp. 11463	+ A. israelii 10215	1.067	0.037	84 ± 10
Veillon. sp. 11463	+ S. salivarius P2	1.135	0.009	425 ± 38
Veillon. sp. 11463	+ S. salivarius 8618	1.150	0.007	472 ± 7
Veillon. sp. 11463	+ S. sanguis P1	1.108	0.011	303 ± 4
Veillon. sp. 11463	+ S. sanguis 7863	1.023	0.011	357 ± 3
A. israelii P2	+ A. israelii 10215	1.135	0.015	237 ± 17
A. israelii P2	+ S. salivarius P2	1.182	0.009	376 ± 47
A. israelii P2	+ S. salivarius 8618	1.196	0.008	399 ± 47
A. israelii P2	+ S. sanguis P1	1.231	0.006	373 ± 26
A. israelii P2	+ S. sanguis 7863	1.154	0.009	409 ± 41
A. israelii 10215	+ S. salivarius P2	1.176	0.011	333 ± 11
A. israelii 10215	+ S. salivarius 8618	1.185	0.012	281 ± 33
A. israelii 10215	+ S. sanguis P1	1.230	0.011	192 ± 2
A. israelii 10215	+ S. sanguis 7863	1.167	0.015	229 ± 11
S. salivarius P2	+ S. salivarius 8618	1.167	0.009	398 ± 22
S. salivarius P2	+ S. sanguis P1	1.130	0.009	338 ± 27
S. salivarius P2	+ S. sanguis 7863	1.084	0.008	414 ± 33
S. salivarius 8618	+ S. sanguis P1	1.170	0.009	316 ± 15
S. salivarius 8618	+ S. sanguis 7863	1.156	0.008	460 ± 45
S. sanguis P1	+ S. sanguis 7863	1.112	0.009	332 ± 14

*$t_{(Ao/2)}$ in minutes ± SEM

Table 5.8 Summary of the $t_{(Ao/2)}$ values from the heterotypic aggregation results.

	S. sanguis 7863	S. sanguis P1	S. salivarius 8618	S. salivarius P2	A. israelii 10215	A. israelii P2	Veillonella sp. 11463	Veillonella sp. P3	Peptostrep. sp. 9807	Peptostrep. sp. P2	H. actinomycetemc. 9710	H. aphrophilus P5	Capnocytophaga sp. 27872	Capnocytophaga sp. P2	B. intermedius 9336	B. intermedius P2	B. gingivalis W83
B. gingivalis P4	142	195	285	221	141	424	457	168	392	448	491	238	190	482	239	125	566
B. gingivalis W83	158	176	283	306	104	337	302	177	600	366	644	240	164	331	299	143	
B. intermedius P2	96	52	77	93	8	146	161	96	197	60	151	54	142	248	215		
B. intermedius 9336	330	250	281	343	208	355	348	162	433	382	356	178	295	307			
Capnocytophaga sp. P2	337	306	457	280	195	375	415	156	565	392	377	217	380				
Capnocytophaga sp. 27872	346	297	389	271	89	259	589	168	416	391	359	182					
H. aphrophilus P5	221	64	136	148	33	167	197	136	257	212	348						
H. actinomycetemc. 9710	416	241	306	383	222	347	306	163	580	471							
Peptostrep. sp. P2	333	281	392	342	179	368	583	186	406								
Peptostrep. sp. 9807	436	347	458	475	311	513	528	196									
Veillonella sp. P3	155	116	37	63	160	186	167										
Veillonella sp. 11463	357	303	472	425	84	410											
A. israelii P2	409	373	399	376	237												
A. israelii 10215	229	192	281	333													
S. salivarius P2	414	338	398														
S. salivarius 8618	460	316															
S. sanguis P1	332																

Table 5.9 Heterotypic aggregation controls in PBS.

Bacterium	Ao	m	$t_{(Ao/2)}$* ± SEM
B. gingivalis P4	1.177	0.012	336 ± 46
B. gingivalis W83	1.160	0.007	478 ± 44
B. intermedius P2	1.212	0.018	180 ± 7
B. intermedius 9336	1.189	0.008	452 ± 57
Capnocytophaga sp. P2	1.173	0.009	376 ± 38
Capnocytophaga sp. 27872	1.094	0.011	508 ± 15
H. aphrophilus P5	1.137	0.028	107 ± 12
H. actinomycetemc. 9710	1.156	0.012	246 ± 27
Peptostrep. sp. P2	1.159	0.008	318 ± 10
Peptostrep. sp. 9807	1.200	0.007	562 ± 12
Veillonella sp. P3	1.184	0.022	163 ± 14
Veillonella sp. 11463	1.175	0.009	353 ± 14
A. israelii P2	1.211	0.009	342 ± 17
A. israelii 10215	1.105	0.017	149 ± 8
S. salivarius P2	1.183	0.012	213 ± 26
S. salivarius 8618	1.157	0.011	313 ± 29
S. sanguis P1	1.176	0.011	239 ± 23
S. sanguis 7863	1.224	0.011	261 ± 38

*$t_{(Ao/2)}$ in minutes.

Table 5.10 Summary of the predicted $t_{(Ao/2)}$ values for different combinations of the heterotypic aggregation controls from Table 5.9.

	S. sanguis 7863	S. sanguis P1	S. salivarius 8618	S. salivarius P2	A. israelii 10215	A. israelii P2	Veillonella sp. 11463	Veillonella sp. P3	Peptostrep. sp. 9807	Peptostrep. sp. P2	H. actinomycetemc. 9710	H. aphrophilus P5	Capnocytophaga sp. 27872	Capnocytophaga sp. P2	B. intermedius 9336	B. intermedius P2	B. gingivalis W83
B. gingivalis P4	297	285	324	269	213	331	344	217	429	328	281	164	426	355	388	236	394
B. gingivalis W83	345	332	377	311	238	392	408	239	520	393	317	177	496	421	464	263	
B. intermedius P2	211	202	230	193	164	228	238	171	287	222	211	134	304	245	264		
B. intermedius 9336	341	329	372	309	239	385	400	240	503	385	315	180	484	412			
Capnocytophaga sp. P2	313	301	341	283	222	351	365	225	457	349	293	169	449				
Capnocytophaga sp. 27872	385	374	411	354	281	426	438	278	529	438	353	215					
H. aphrophilus P5	148	142	162	137	123	157	164	131	193	151	155						
H. actinomycetemc. 9710	252	243	273	232	193	274	285	198	342	270							
Peptostrep. sp. P2	285	272	315	255	199	322	337	204	432								
Peptostrep. sp. 9807	378	365	410	342	260	429	444	259									
Veillonella sp. P3	196	188	213	181	157	210	218										
Veillonella sp. 11463	302	290	331	273	215	339											
A. israelii P2	290	278	318	261	206												
A. israelii 10215	190	182	209	174													
S. salivarius P2	236	225	261														
S. salivarius 8618	287	276															
S. sanguis P1	250																

The heterotypic aggregation results in Table 5.8 were compared to the predicted control results in Table 5.10 to determine if significant aggregation resulted in the test suspensions. To simplify this comparison, the reduction in the $t_{(Ao/2)}$ values of the tests were represented as a percentage reduction compared to the predicted non-aggregating control results. These percentage results are summarized in Table 5.11.

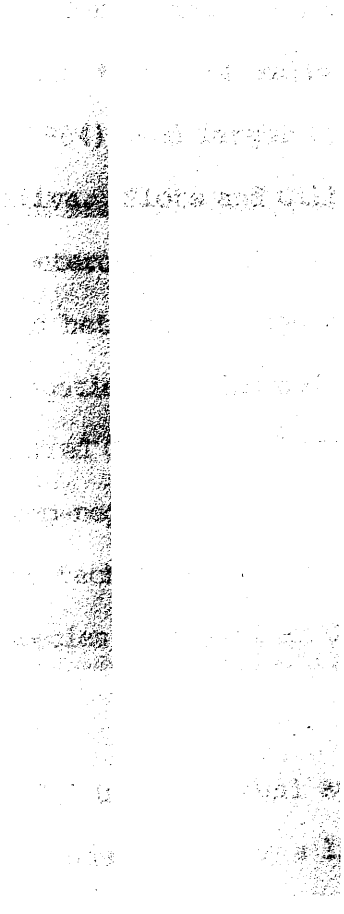
The percentage decreases given in Table 5.11 range from 0 to 95 per cent, relating to zero and rapid heterotypic aggregation, respectively. Marked aggregation was considered subjectively to be a reduction of greater than 20 per cent, and approximately one third of the 153 bacterial pairs tested were in this category. Of these, approximately half (one sixth of the total) demonstrated rapid aggregation with reductions of greater than 40 per cent.

The distribution of aggregating pairs gave certain discernible patterns. The most noticable was the complete absence of aggregation between any of the eight Gram-positive bacteria with any other Gram-positive bacterium. The strains of bacteria giving the most aggregation were B. intermedius P2 and Veillonella species P3, which aggregated with 15 and 12 other bacteria, respectively. Because of the wide spectrum of aggregating activity of these two bacteria, the definition of aggregating groups is less clear. However, two general groups of aggregating bacteria were still evident. One group consisted of the Bacteroides and Capnocytophaga species where most of these Gram-negative bacteria aggregated with each other. The other group consisted of the Bacteroides and Capnocytophaga species which aggregated with most of the Streptococcus and Actinomyces species.

Table 5.11 Summary of the percentage reductions of the $t_{(Ao/2)}$ values of the tests (Table 5.8) compared with the $t_{(Ao/2)}$ values of the controls (Table 5.10).

	S. sanguis 7863	S. sanguis P1	S. salivarius 8618	S. salivarius P2	A. israelii 10215	A. israelii P2	Veillonella sp. 11463	Veillonella sp. P3	Peptostrep. sp. 9807	Peptostrep. sp. P2	H. actinomycetemc. 9710	H. aphrophilus P5	Capnocytophaga sp. 27872	Capnocytophaga sp. P2	B. intermedius 9336	B. intermedius P2	B. gingivalis W83
B. gingivalis P4	52	32	12	18	34	0	0	23	9	0	0	0	55	0	38	47	0
B. gingivalis W83	54	47	25	2	56	14	26	26	0	7	0	0	67	21	36	46	
B. intermedius P2	55	74	67	52	95	36	32	44	31	73	28	60	53	0	19		
B. intermedius 9336	3	24	24	0	13	8	13	32	14	1	0	1	39	25			
Capnocytophaga sp. P2	0	0	0	1	12	0	0	31	0	0	0	0	15				
Capnocytophaga sp. 27872	10	21	5	23	68	39	0	40	21	9	0	15					
H. aphrophilus P5	0	55	16	0	73	0	0	0	0	0	0						
H. actinomycetemc. 9710	0	1	0	0	0	0	0	18	0	0							
Peptostrep. sp. P2	0	0	0	0	10	0	0	9	6								
Peptostrep. sp. 9807	0	5	0	0	0	0	0	24									
Veillonella sp. P3	21	38	83	64	0	11	24										
Veillonella sp. 11463	0	0	0	0	61	0											
A. israelii P2	0	0	0	0	0												
A. israelii 10215	0	0	0	0													
S. salivarius P2	0	0	0														
S. salivarius 8618	0	0															
S. sanguis P1	0																

In a comparison of the fresh and type strains, no significant differences could be noted in the relative amounts of aggregation. In addition, none of the fresh strains produced aggregation patterns similar to the type strains of the same species, with the possible exception of the B. gingivalis strains, which gave similar results with 13 of the 16 possible comparisons. However, certain similarities appear to exist between the two strains of each species, because none of the fresh strains aggregated with the type strains of the same species, with the exception of the Veillonella strains P3 and 11463.



5.4 DISCUSSION

5.4.1 Experimental method

The aggregation of oral bacteria has been investigated by a number of workers. In an *in vivo* study, Liljemark and Gibbons (1971) introduced streptomycin resistant *Veillonella* species, *Neisseria* species, *S. sanguis* and *S. salivarius* into the mouths of volunteers. They observed that *S. sanguis* adhered well to pre-formed dental plaques better than the other bacteria tested. Gibbons and Nygaard (1970) used an *in vitro* method to assess bacterial aggregation with 0.3 ml volumes of bacterial suspensions which were incubated and then assessed for aggregation visually using a dissecting microscope, scoring aggregation as 0 to 4+. Morris and McBride (1983) used this technique to determine aggregation titers of saliva with strains of *S. sanguis*, and Cisar *et al.* (1984) used larger volumes to measure heterotypic aggregation in saliva. Slots and Gibbons (1978) used a similar method to assess both heterotypic and homotypic aggregation. However, they could not assess heterotypic aggregation by direct visualisation as homotypic aggregation was induced by the suspending medium. Consequently, they prepared Gram-stained smears of aggregates to assess the attachment of Gram-negative to Gram-positive bacteria microscopically. Clearly this technique cannot be used to assess aggregation between bacterial species with similar Gram-reactions and morphologies.

Bourgeau and McBride (1976) used a visual aggregation assay similar to those above, but concluded that only a limited amount of information could be obtained from such assays. They therefore

developed, a more accurate method in which suspensions of bacteria were left to aggregate and settle at the bottom of test tubes. The number of bacteria in the original suspension and in the supernatant were determined by cultural methods and thereby the proportion of bacteria which aggregated was calculated. This method was modified by McIntire et al. (1978), who centrifuged aggregated suspensions at a low speed and determined the amount of aggregation by measuring the absorbance of the supernatant.

A more accurate method was developed by Ericson et al. (1975) using a spectrophotometric method, on which the methods used in this study were based. Cisar et al. (1979) compared Ericson's method of monitoring the absorbance changes of a bacterial suspension spectrophotometrically, to a method similar to that used by Gibbons and Nygaard (1970) where aggregation was scored visually from 0 to 4+. Cisar found that aggregation reactions that appeared to be unaffected by the addition of lactose in the visual assay were subsequently shown to be inhibited by up to 60 per cent using the spectrophotometric method. Therefore, it appears that Ericson's method is preferable because of the sensitivity of this technique.

Golub et al. (1979) compared Ericson's method with two other methods designed to give similar levels of accuracy. The first method involved centrifuging an aggregated suspension and determining the number of bacteria in the supernatant and pellet by using radioactively labelled bacteria. The second method, also using radiolabelled bacteria, separated aggregated bacteria by filtration and determined the numbers of bacteria in the filtrate and on the filter. Golub and co-workers concluded that these methods were faster

and required less saliva and bacteria than Ericson's method, however they were also less reproducible and provided less information on parameters such as aggregation rates.

A possible criticism of Ericson's method is that it is not rapid enough for use with large numbers of samples, but because of the accuracy and reproducibility of this method, it was considered to be preferable for use in this study. To make the assay less labour intensive, up to four cuvettes at a time were monitored automatically over the test period and the data analysis described by Ericson was computerized, thereby considerably shortening the time and effort required for the compilation of results.

5.4.2 Aggregation and absorbance

The data obtained formed a sigmoidal curve, the nature of which can be explained as follows. The individual cells present in the suspension at the beginning of the reaction are effective light scattering bodies, by virtue of their size in relation to the wavelength of light used. This gives the suspension a high measured absorbance. The proceeding formation of small aggregates of bacterial cells produces bodies which are larger, and therefore less effective light scattering centres and consequently the absorbance falls. The formation of these small, non-sedimenting aggregates, results in a gradual decrease in absorbance of the suspension (Koch, 1984). This is followed by a more rapid decrease in absorbance due to the formation of larger aggregates, which scatter light even less effectively, but also begin to settle out of suspension. Finally this rapid decrease is retarded at lower absorbancies by non-aggregated

cells and cell debris remaining in suspension and by the adherence of aggregates to the walls of the cuvette.

It is also possible that a decrease in absorbance is due to the lysis of bacterial cells in the suspension. To determine if this occurred, at the end of the assay period aggregates were dispersed by vortex mixing, and the absorbance was compared with the starting absorbance. In every case any differences were negligible, or were due to incomplete dis-aggregation.

5.4.3 Homotypic aggregation

Six of the bacteria studied aggregated in SIB, with $t_{(Ao/2)}$ values between 21 and 61 minutes, but none of the bacteria tested aggregated markedly in PBS (Table 5.5). The divalent ion, calcium (which is present in SIB but not PBS) has been implicated in the aggregation of certain bacteria and may be responsible for the differences in the two buffers. For example, homotypic aggregation of S. mutans serotype c (Rundegren and Ericson, 1981c) and a dental plaque cocco-bacillus (Gibbons and Spinnell, 1969) were shown to be dependent on calcium. Alternatively, divalent magnesium ions or the relatively high concentration of potassium chloride present in SIB may be involved. However, no previous reports have alluded to this possibility and calcium ions appear to be the most probable cause of SIB induced aggregation.

Twelve strains aggregated in saliva (Table 5.5), including all four Streptococcus strains. This agrees with numerous reports on the aggregation of S. sanguis, S. mutans and S. mitior in this secretion

(Kashket and Donaldson, 1972; McBride and Gisslow, 1977; Levine et al., 1978; Kashket et al., 1982; Murray et al., 1982; Rosan et al., 1982b) and it has also been reported that 41 per cent of S. salivarius isolates aggregated in saliva (Weerkamp and McBride, 1980a). Salivary aggregation may therefore be a factor of some importance in the colonization and formation of dental plaques by S. sanguis, and bacterial accumulations on the dorsum of the tongue by S. salivarius.

In contrast, A. israelii is indigenous to dental plaque (Bowden et al., 1975; Loesche and Syed, 1978) but both strains tested failed to aggregate in saliva. In addition, only the type Veillonella strain aggregated in saliva, although this property might be expected to enhance the colonizing ability of this species commonly found on the tongue dorsum and in plaque (Liljemark and Gibbons, 1971; Bowden et al., 1975). These results largely agree with the findings of Socransky et al. (1977) who reported that Veillonella species and A. israelii did not aggregate in saliva. This suggests that saliva induced aggregation is not an important factor in the colonization of the oral cavity by these bacteria.

Surprisingly little information exists in the literature on saliva induced aggregation of bacteria implicated in periodontal disease. In this study, 70 per cent of such bacteria aggregated in saliva suggesting that this property may influence their colonization, and may therefore be an area of research that deserves further study. In addition to affecting colonization, salivary aggregation may also aid the formation and maintenance of tenacious bacterial deposits at the gingival margin or even in the gingival crevice. However, it is likely that bacteria in the gingival crevice may rarely be exposed to

saliva. Therefore, in this situation aggregating components in crevicular fluid may serve a similar role; for example, a high molecular weight glycoprotein was shown to be present in both saliva and crevicular fluid that aggregated S. sanguis (Morris and McBride, 1983).

A comparison of the salivary aggregation results with the adherence results from Chapters 2, 3 and 4 is presented in Table 5.12. A measure of the ability of the test bacteria to aggregate in saliva was obtained by calculating the percentage decrease that occurred in PBS plus saliva compared with PBS alone. No significant correlations ($p > 0.1$) were found between the adherence and aggregation results. In addition, adherence of the fresh strains was predominantly better than the type cultures, with the exception of the Peptostreptococcus species, but this pattern was not evident with salivary aggregation. The fresh strains aggregated better than the type strains, but with the exception of Veillonella species, A. israelii and S. salivarius. Thus it appears that saliva induced aggregation and adherence to oral surfaces are independent. This conclusion agrees with the findings of Rosan et al. (1982b) who reported that the adherence of S. sanguis, S. mitior and S. mutans to hydroxyapatite and the aggregation of these organisms are mediated by distinct salivary components. However, Ericson and Magnusson (1976) noted that the aggregating factors for these Streptococcus species showed a distinct affinity for hydroxyapatite. The weight of evidence though, appears to indicate that adherence and aggregation are mediated by different salivary factors.

Table 5.12 Bacterial adherence to buccal cells (Table 2.4*), saliva treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), compared with bacterial aggregation in saliva (Table 5.5+).

Bacterium	Bacteria per 0.018 mm²			% reduction
	buccal cells	saliva treated Hela cells	saliva treated enamel	saliva induced aggregation
B. gingivalis P4	252	100	128	34
B. gingivalis W83	19	16	30	0
B. intermedius P2	38	8	336	23
B. intermedius 9336	8	0	7	0
Capnocytophaga sp. P2	30	4	79	73
Capnocytophaga sp. 27872	32	1	67	74
H. aphrophilus P5	68	15	24	58
H. actinomycetemc. 9710	64	93	324	0
Peptostrep. sp. P2	119	127	41	86
Peptostrep. sp. 9807	445	313	225	44
Veillonella sp. P3	709	139	584	0
Veillonella sp. 11463	176	88	151	93
A. israelii P2	14	16	24	16
A. israelii 10215	3	0	0	29
S. salivarius P2	309	397	47	68
S. salivarius 8618	22	325	22	91
S. sanguis P1	238	268	197	91
S. sanguis 7863	7	110	9	78

*Buccal cell results calculated as in Section 3.2.8.

+percentage reduction in PBS plus saliva compared to PBS.

The $t_{(Ao/2)}$ values of the fresh strains (Table 5.5) were predominantly significantly lower ($p < 0.05$) than the type strains in the four suspending media, with the exception of the A. israelii strains. Repeated subculturing may therefore inhibit the subsequent homotypic aggregation properties of isolates. However, the aberrant organisms that demonstrated better aggregation and adherence with the type strains were of different species; as was the case with salivary aggregation. Therefore, the factors involved in homotypic aggregation appear to be distinct from those mediating adherence to oral surfaces.

5.4.4 Heterotypic aggregation

The predicted control $t_{(Ao/2)}$ values were calculated assuming that separate control suspensions act in the same way as mixed suspensions of two bacteria. The original absorbancies and experimental methods used were the same, but the cell concentrations of each bacterium were half that in the controls because the two suspensions dilute each other when mixed. Theoretically, if no homotypic aggregation occurs in the test buffer, this factor should have no significant effect because the total cell concentration will be the same as the controls. Indeed, the $t_{(Ao/2)}$ values of the controls were high, indicating little or no homotypic aggregation, but the heterotypic aggregation results suggested that a measurable degree of homotypic aggregation did occur in some cases. This was shown where the $t_{(Ao/2)}$ values of the mixed test suspensions were higher than the predicted control results, ie. less aggregation occurred in the test suspension. For this to happen, a degree of homotypic aggregation in PBS with one or both organisms had to occur and there

also had to be little or no heterotypic aggregation between the two test bacteria. Then the diluting effect of one suspension on the other may result in a partial inhibition of homotypic aggregation.

Preliminary experiments indicated that altering the original absorbancies affected the resulting $t_{(Ao/2)}$ values. Therefore, it was not possible to use control suspensions with the same cell concentration as the individual strains in the tests. Consequently the predicted mixed control $t_{(Ao/2)}$ values were not corrected to account for homotypic aggregation in PBS. However, homotypic aggregation in PBS was slight or absent, and did not appear to substantially influence interpretation of heterotypic aggregation. For example, H. aphrophilus P5 and A. israelii 10215 gave the lowest control $t_{(Ao/2)}$ values of the bacteria tested, but a mixture of the two bacteria produced a 73 per cent decrease in the $t_{(Ao/2)}$ value compared to the predicted control value.

Intraoral aggregation interactions occur mainly in saliva, although the heterotypic aggregation assays could not be performed in this secretion in the present study because of the wide occurrence of saliva induced aggregation with the bacteria used. However, according to Slots and Gibbons (1978) and Kolenbrander and Phucas (1984), heterotypic aggregation is largely unaffected by suspending the test bacteria in either buffer or saliva. This suggests that the present in vitro findings are comparable to interactions which occur in the in vivo environment.

The most noticeable feature of the heterotypic aggregation results was the lack of aggregation amongst any of the Gram-positive bacteria, as illustrated in Table 5.13 (abstracted from Table 5.11). In agreement with this study was the report of Gibbons and Nygaard (1970) who tested heterotypic aggregation between four S. sanguis, four S. salivarius and six S. mutans isolates, giving 91 pairs. Only two pairs showed very weak aggregation and the remaining pairs showed none. Taking into account the ability of the streptococci tested to aggregate in saliva, this study indicates that the major factor in the formation of plaques predominated by streptococci is saliva induced homotypic aggregation.

In contrast, many reports have studied aggregation between A. naeslundii and A. viscosus with S. sanguis, S. mitior or S. mutans (McBride and Bourgeau, 1975; Ellen and Balcerzak-Raczkowski, 1977; Cisar et al., 1979 and 1980; Kolenbrander and Williams, 1981 and 1983; Mizuno et al., 1983; Kolenbrander and Phucas, 1984). These reports have determined that a large proportion of streptococci aggregate with these Actinomyces species and the mechanisms involved have been partially charaterized. However, Kolenbrander and Celesk (1983) reported that A. israelii, although of the same genus, appears to differ from A. naeslundii and A. viscosus in that A. israelii aggregated with Capnocytophaga species while the latter species did not. The studies of Kolenbrander and Celesk (1983) and Cisar et al. (1979) also indicated that A. israelii may differ from A. naeslundii and A. viscosus in not aggregating with S. salivarius or S. sanguis. It is possible that in the present study the removal of cell aggregates of A. israelii from the broths in preparing test

Table 5.13 Heterotypic aggregation between the Gram-positive bacteria (percentage reductions in $t_{(Ao/2)}$ values).

	S. sanguis 7863	S. sanguis P1	S. salivarius 8618	S. salivarius P2	A. israelii 10215	A. israelii P2	Peptostrep. sp. 9807
Peptostrep. sp P2	0	0	0	0	10	0	6
Peptostrep. sp. 9807	0	5	0	0	0	0	
A. israelii P2	0	0	0	0	0		
A. israelii 10215	0	0	0	0			
S. salivarius P2	0	0	0				
S. salivarius 8618	0	0					
S. sanguis P1	0						

suspensions may have removed the cells most able to aggregate, but both A. israelii strains aggregated strongly with some of the Gram-negative bacteria, so this seems unlikely. Therefore, the results of this study tend to support the idea that A. israelii differs from A. naeslundii and A. viscosus in its ability to aggregate with streptococci.

Unlike the Gram-positive bacteria tested, the Gram-negative isolates did aggregate with each other, with most aggregation occurring between the Bacteroides and Capnocytophaga species as shown in Table 5.14. Most heterotypic aggregation studies in the past have paired Gram-negative bacteria with Gram-positives and so may overlook this phenomenon, although two recent reports have investigated the aggregation of three strains of B. intermedius and a B. gingivalis strain against a number of Capnocytophaga strains (Kolenbrander and Andersen, 1984; Kolenbrander, Andersen and Holdeman, 1985). However, no aggregation was noted with any of the pairs tested, so these results are clearly at variance with those of the present study. This could be due to differences in the experimental methods used. For example, the above studies used a method which determined the presence of bacterial aggregates in a suspension by direct visualization, although Bourgeau and McBride (1976) concluded that such a method could yield only a limited amount of information. However, the amounts of aggregation recorded in this study should be sufficient to register in the visual assay used by Kolenbrander's group. Therefore, the discrepancies noted are more probably due to strain differences, or possibly to the different buffers used.

Table 5.14 Heterotypic aggregation with the Bacteroides and Capnocytophaga species (percentage reductions in $t_{(Ao/2)}$ values).

	Capnocytophaga sp. 27872	Capnocytophaga sp. P2	B. intermedius 9336	B. intermedius P2	B. gingivalis W83
B. gingivalis P4	55	0	38	47	0
B. gingivalis W83	67	21	36	46	
B. intermedius P2	53	0	19		
B. intermedius 9336	39	25			
Capnocytophaga sp. P2	15				

The other main aggregation group occurred with B. gingivalis, B. intermedius and C. ochraceus which aggregated with S. salivarius, S. sanguis and A. israelii (Table 5.15). These results agreed with those of Slots and Gibbons (1978) who reported similar results with these species. Also, Weerkamp and McBride (1980a) noted that B. gingivalis aggregated with S. salivarius and Kolenbrander et al. (1985) showed that B. intermedius aggregated with A. israelii. However, the latter report also noted that B. intermedius did not aggregate with A. israelii or S. sanguis, which is at variance with the present results. In addition, Kolenbrander and Celesk (1983) found that with nineteen strains of Capnocytophaga species mixed with eight strains of A. israelii, only 26 per cent of the pairs aggregated, and the Capnocytophaga species did not aggregate with with strains of S. salivarius or S. sanguis. Therefore, the current literature is somewhat contradictory, which may be due to the use of different bacterial strains or methods, and perhaps illustrates the need for a standard assay. However, the majority of reports are in agreement with the findings of this study.

The ability of the Gram-negative bacteria to aggregate heterotypically with each other and with Gram-positive bacteria commonly found in the oral cavity may be important factors governing their colonization in vivo. This may be particularly so in view of the fact that the Bacteroides and Capnocytophaga species generally adhered poorly to the various oral surfaces tested earlier in this thesis, and that only the Capnocytophaga species aggregated markedly in saliva (Table 5.12). Heterotypic aggregation may therefore be the main mechanism by which these bacteria adhere in the oral cavity.

Table 5.15 Heterotypic aggregation between the Bacteroides and Capnocytophaga species with the Streptococcus and Actinomyces species (percentage reductions in $t_{(Ao/2)}$ values).

	S. sanguis 7863	S. sanguis P1	S. salivarius 8618	S. salivarius P2	A. israelii 10215	A. israelii P2
B. gingivalis P4	52	32	12	18	34	0
B. gingivalis W83	54	47	25	2	56	14
B. intermedius P2	55	74	67	52	95	36
B. intermedius 9336	3	24	24	0	13	8
Capnocytophaga sp. P2	0	0	0	1	12	0
Capnocytophaga sp. 27872	10	21	5	23	68	39

In this study few differences were noted between the heterotypic aggregation results of the fresh and type strains, although the fresh strains aggregated marginally better. In contrast, adherence to oral surfaces and homotypic aggregation of the type strains were predominantly less pronounced than that shown by the fresh strains. In addition, there were no significant correlations ($p > 0.1$) between the relative amounts of heterotypic aggregation of the various strains tested and adherence or homotypic aggregation in saliva. These observations suggest that the factors involved in heterotypic aggregation are different from those mediating adherence or saliva induced homotypic aggregation.

5.5 CONCLUSIONS

The aggregation method used gave a degree of accuracy and reproducibility superior to that reported for alternative methods. Although this method was not as rapid as some developed by other workers, it was simplified by automating part of the assay and computerising the analysis of the data.

Saliva aggregated 60 per cent of the bacteria tested and appears to be mediated by components distinct from those involved in adherence or heterotypic aggregation. All of the streptococci tested aggregated in saliva indicating that salivary aggregation may be important in the formation of streptococcal plaques.

Heterotypic aggregation did not occur between any of the Gram-positive bacteria tested. Instead, aggregation occurred mainly with: (i) the Bacteroides and Capnocytophaga species which aggregated with each other, and (ii) the Bacteroides and Capnocytophaga species which aggregated with the Streptococcus and Actinomyces species. Heterotypic aggregation may be the major factor governing the oral colonization of Bacteroides and Capnocytophaga species, and appears to be mediated by factors distinct from those involved in adherence or salivary aggregation. Heterotypic aggregation of the bacteria thought to be implicated in periodontal disease may therefore merit further study.

CHAPTER 6

DETERMINATION OF BACTERIAL HYDROPHOBICITY

6.1 INTRODUCTION

The importance of bacterial cell surface hydrophobicity in adherence has been reported by many research groups (Rosenberg et al., 1983b). However, a number of reports have concluded that hydrophobic interactions are of little importance (Rosan et al., 1985) and consequently opinion is divided on the subject.

It can be postulated that if the adherence of microorganisms is mediated by hydrophobic factors, then a clear association between adherence and hydrophobicity should be evident. Previous reports linking hydrophobicity to adherence have found a correlation between the hydrophobicities of a number of bacteria and their ability to adhere to one type of surface (Perers et al., 1977; Rosenberg et al., 1981; Gibbons and Ethereden, 1983; Gibbons et al., 1983b; Fives-Taylor and Thompson, 1985). If hydrophobic interactions are the main factors governing adherence, then specific interactions with different oral surfaces will not be important and adherence will always correlate with the hydrophobic potentials of the bacteria. Therefore, this study aimed to assess the hydrophobicity of the 18 bacteria studied in the previous chapters and to determine if there was any relationship between hydrophobicity and adhesion or aggregation.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of bacterial suspensions

The bacteria detailed in Sections 2.2.1 to 2.2.5 were grown as described in Section 2.2.6. The cultures were centrifigued at 3000 g for 10 minutes in sterile disposable plastic universals (Nunc Inter Med., Kamstrup, Denmark) in an MSE super minor centrifuge (MSE Scientific Instruments, Crawley, England). The resultant pellet was resuspended in either 20 ml of saliva ions buffer (SIB) (Appendix 8) or 20 ml of phosphate buffered saline (PBS) (Appendix 9) and centrifuged again at 3000 g for 10 minutes. The bacterial pellet was then resuspended in either SIB, PBS, clarified saliva diluted 1 in 6 with SIB or clarified saliva diluted 1 in 6 with PBS. Whole, mixed, unstimulated saliva was collected and clarified as in Section 3.2.4.

The bacterial suspensions were then diluted with the appropriate suspending medium to give an optical density of 1.0 (± 0.02) at a wavelength of 520 nm, using plastic disposable cuvettes with a path length of 10 mm (Sarstedt Ltd., Leicester, England), in an SP 8-100 spectrophotometer (Pye Unicam, Cambridge, England), which resulted in suspensions containing between approximately $2x10^8$ to $5x10^9$ bacteria per ml.

6.2.2 Hydrophobicity assay procedure

The assay used was based on the method described by Rosenberg, Gutnick and Rosenberg (1980). This is a relatively simple assay measuring the affinity of bacteria for an immiscible liquid

hydrocarbon, such as xylene, mixed with an aqueous suspension of bacteria. On mixing, hydrophobic bacteria will adhere to the xylene, and as the two immiscible phases separate, bacteria with a hydrophobic surface will be removed from the aqueous suspension. Hydrophilic bacteria will remain in the aqueous phase. The absorbance of the aqueous suspension is then measured and compared to a control suspension without xylene. A diagrammatic representation of the assay procedure is shown in Figure 6.1.

The composition of the aqueous suspension can significantly affect the hydrophobic potentials of the test bacteria (Nesbitt et al., 1982a; Rogers et al., 1984). Therefore, to relate the hydrophobicity results obtained to the adherence results reported earlier, SIB was used as the suspending medium. In addition, SIB plus saliva, PBS and PBS plus saliva were used for comparisons with the aggregation data from Chapter 5.

The assay was performed in 15 mm diameter, 150 mm long, round-bottomed glass test tubes, cleaned in a Miele Electronic G 192 washer (Miele Co. Ltd., Abingdon, England) using de-ionised water. For each test bacterium in each suspending medium, 5 ml of bacterial suspension was added to each of two test tubes, giving one test and one control. In addition, a test and a control of the suspending medium alone were prepared in order to provide zero controls for the spectrophotometer. Xylene (Raymed, Leeds, England) was then added carefully down the inner wall of one of the pair of test tubes so that mixing did not occur and the xylene formed a layer floating on top of the bacterial suspension. Nothing was added to the control bacterial suspensions. The test tubes were then incubated in a water bath (A. Gallenkamp and

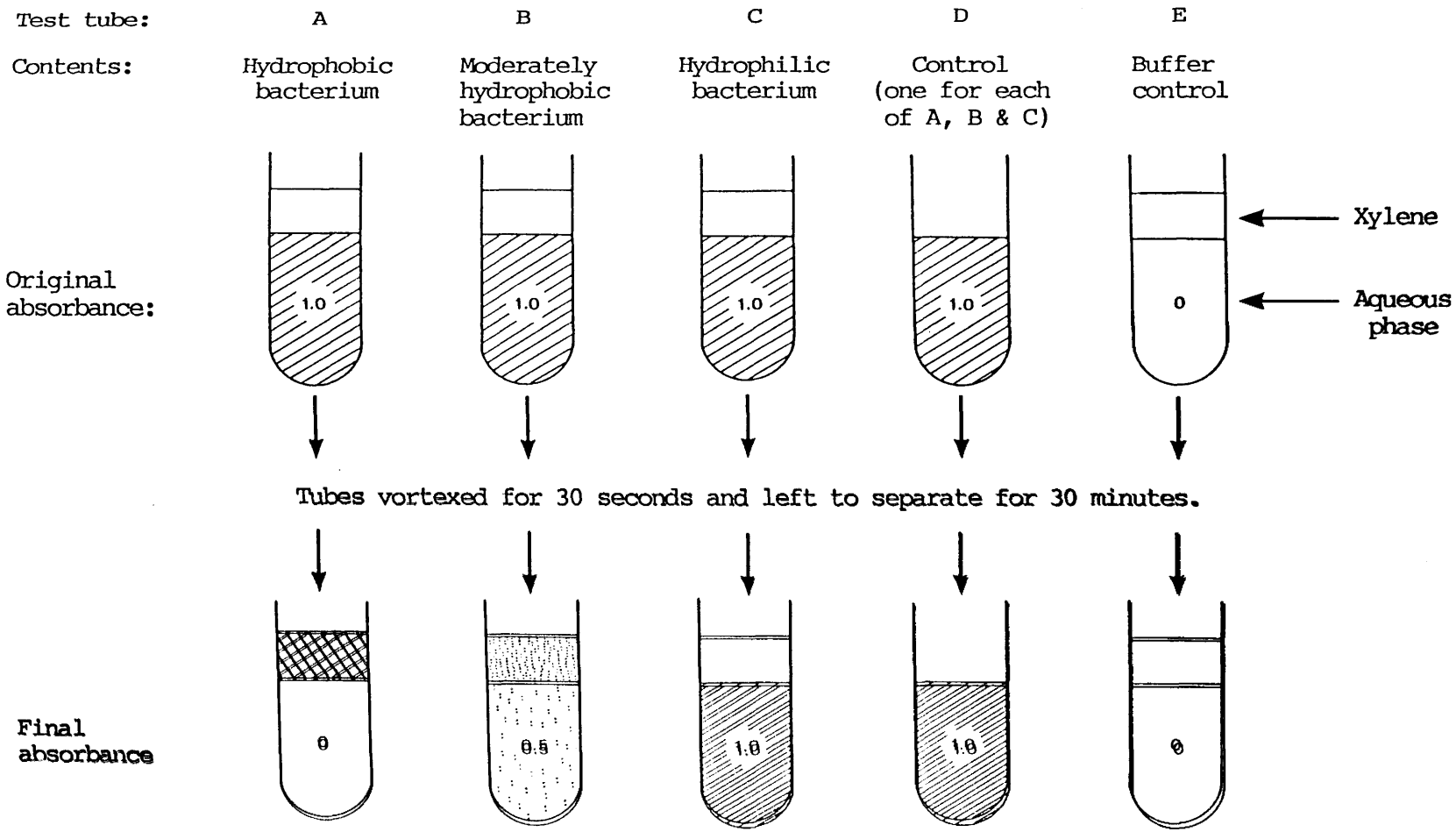


Figure 6.1 The hydrophobicity assay procedure.

Co., East Kilbride, Glasgow, Scotland) at 37°C for 10 minutes, after which time the temperature of the suspensions had equilibrated to that of the water bath.

Following the 10 minute pre-incubation period, all of the test tubes were taken in turn and uniformly mixed on a Fisons whirlimixer for 30 seconds (Fisons Scientific Apparatus, Loughborough, Leicestershire, England). After mixing, the tubes were returned to the water bath for 30 minutes. During this period, the xylene added to each test suspension separated out from the aqueous phase and rose to form a separate layer (Figure 6.2). The lower aqueous layer was then carefully removed to a clean test tube using a glass pasteur pipette. This was achieved by carefully passing the pipette tip through the xylene layer into the aqueous suspension, expelling two or three bubbles of air to ensure that no xylene was in the pipette and then aspirating the aqueous layer. After withdrawing the pipette, the first few drops, which could contain xylene, were discarded and the remainder was expelled into a clean test tube.

Finally, any contaminating xylene that may have been carried over in the pipette or bound to the bacterial cells was removed. To accomplish this, air from the laboratory compressed air supply was bubbled through the suspensions with a clean pasteur pipette for two minutes at a rate of approximately 3 ml per second. This resulted in the evaporation of any contaminating xylene by virtue of its volatile nature.

Tubes were then vortex mixed for five seconds to resuspend any cells that had aggregated or settled out, and the final optical

Figure 6.2 Hydrophobicity assay tubes.

Tube 1: a control bacterial suspension without xylene.

Tube 2: hydrophilic bacteria unaffected by the xylene and remaining in the aqueous suspension after vortex mixing.

Tube 3: moderately hydrophobic bacteria divided between the xylene and the aqueous layers making both slightly turbid.

Tube 4: hydrophobic bacteria removed from the aqueous suspension by the xylene leaving a clear aqueous phase.

densities of the suspensions were determined at a wavelength of 520 nm in the SP 8-100 spectrophotometer. The suspending medium control with xylene added was used to zero the spectrophotometer for reading the optical densities of the xylene added test suspensions. The suspending medium control without xylene was used to zero the spectrophotometer for the control suspensions.

Hexadecane (BDH Chemicals Ltd., Poole, England) was used in place of xylene on approximately twenty occasions. Experiments were carried out using basically the same methods as described above for xylene.

6.2.3 Calculation of hydrophobicity values

To determine the hydrophobicity of the test bacteria, the percentage reduction in optical density of the test suspensions (with xylene) as compared with the control suspensions (without xylene) were calculated. These percentage values are proportional to the hydrophobicity of the test bacteria in any given suspending medium (Rosenberg et al., 1980).

6.2.4 Statistical analyses

The assay procedure was performed a minimum of three times for each bacterium under study. The mean hydrophobicity values and the standard errors of the means were calculated using standard statistical formulae. The significance of differences between strains were determined using the Mann-Whitney U test, and between sets of data obtained with different buffers using the Wilcoxon matched pairs

signed-rank test. Correlation coefficients were determined for comparisons of the hydrophobicity results with the adherence and aggregation results from the previous chapters.

6.3 RESULTS

The assay was used satisfactorily with all of the bacteria tested and the results are listed in Tables 6.1 to 6.4 and are summarized in Table 6.5. The percentage reductions in absorbance of the test suspensions compared with the controls ranged from 0 to 100 per cent. Most of the bacteria in the different suspending media demonstrated a hydrophobic tendency, with the exception of A. israelii P2 and S. sanguis 7863. More than three quarters of the results showed decreases of greater than 50 per cent.

The hydrophobic properties of the bacteria were shown to be markedly affected by the suspending media. Reference to the mean hydrophobic values for each medium (Table 6.5) showed that bacteria suspended in SIB gave the highest values, and PBS the lowest. The addition of saliva to SIB caused a significant decrease in hydrophobicity compared with SIB alone ($p < 0.01$). No overall significant differences were noted between SIB diluted saliva, PBS or PBS diluted saliva.

Eight pairs of fresh and type strains of different species (excluding the Haemophilus species) were compared in four suspending media giving 32 pairs of test results. The results showed that with 12 pairs the fresh strains were more hydrophobic ($p < 0.05$), with 10 pairs the converse was true ($p < 0.05$) and with the other 10 pairs there were no significant differences ($p > 0.05$). Thus, the distribution of hydrophobic strains does not appear to be influenced by the use of fresh or type strains.

Table 6.1 Hydrophobicity of bacteria suspended in SIB.

Bacterium	percentage reduction in absorbance of aqueous phase after mixing with xylene			
	experiment number			
	1	2	3	mean ± SEM
B. gingivalis P4	95	96	99	97 ± 1.2
B. gingivalis W83	75	74	77	75 ± 0.9
B. intermedius P2	98	98	99	98 ± 0.3
B. intermedius 9336	85	92	88	88 ± 2.0
Capnocytophaga sp. P2	76	72	82	77 ± 2.9
Capnocytophaga sp. 27872	89	83	88	88 ± 2.9
H. aphrophilus P5	84	91	82	86 ± 2.7
H. actinomycetemc. 9710	91	91	85	89 ± 2.0
Peptostrep. sp. P2	98	99	99	99 ± 0.3
Peptostrep. sp. 9807	99	100	100	100 ± 0.3
Veillonella sp. P3	99	99	100	99 ± 0.3
Veillonella sp. 11463	99	100	96	98 ± 1.2
A. israelii P2	95	93	89	92 ± 1.8
A. israelii 10215	81	85	93	86 ± 3.5
S. salivarius P2	99	98	100	99 ± 0.6
S. salivarius 8618	98	99	92	96 ± 2.2
S. sanguis P1	99	99	99	99 ± 0.0
S. sanguis 7863	62	52	73	62 ± 6.1

Table 6.2 Hydrophobicity of bacteria suspended in SIB plus saliva.

Bacterium	percentage reduction in absorbance of aqueous phase after mixing with xylene			
	experiment number			
	1	2	3	mean ± SEM
B. gingivalis P4	92	99	97	96 ± 2.1
B. gingivalis W83	64	72	66	67 ± 2.4
B. intermedius P2	78	67	79	75 ± 3.8
B. intermedius 9336	78	72	77	76 ± 1.9
Capnocytophaga sp. P2	59	56	66	60 ± 3.0
Capnocytophaga sp. 27872	83	89	87	86 ± 1.8
H. aphrophilus P5	79	75	78	77 ± 1.2
H. actinomycetemc. 9710	89	82	90	87 ± 2.5
Peptostrep. sp. P2	42	44	45	44 ± 0.9
Peptostrep. sp. 9807	64	53	55	57 ± 3.4
Veillonella sp. P3	90	94	90	91 ± 1.3
Veillonella sp. 11463	61	66	52	60 ± 4.1
A. israelii P2	13	4	15	11 ± 3.4
A. israelii 10215	68	79	72	73 ± 3.2
S. salivarius P2	59	72	66	66 ± 3.7
S. salivarius 8618	74	73	67	71 ± 2.2
S. sanguis P1	94	96	98	96 ± 1.2
S. sanguis 7863	0	0	1	0 ± 0.3

Table 6.3 Hydrophobicity of bacteria suspended in PBS.

Bacterium	percentage reduction in absorbance of aqueous phase after mixing with xylene			
	experiment number			
	1	2	3	mean ± SEM
B. gingivalis P4	63	80	68	70 ± 5.0
B. gingivalis W83	57	61	63	60 ± 1.8
B. intermedius P2	32	35	33	33 ± 0.9
B. intermedius 9336	48	51	52	50 ± 1.2
Capnocytophaga sp. P2	37	40	31	36 ± 2.6
Capnocytophaga sp. 27872	67	63	49	60 ± 5.5
H. aphrophilus P5	59	67	67	64 ± 2.7
H. actinomycetemc. 9710	87	91	80	86 ± 3.2
Peptostrep. sp. P2	69	59	66	65 ± 3.0
Peptostrep. sp. 9807	57	65	58	61 ± 2.5
Veillonella sp. P3	34	36	37	36 ± 0.9
Veillonella sp. 11463	75	61	65	67 ± 4.2
A. israelii P2	10	13	22	15 ± 3.6
A. israelii 10215	5	4	1	3 ± 1.2
S. salivarius P2	81	85	83	83 ± 1.2
S. salivarius 8618	90	88	93	90 ± 1.5
S. sanguis P1	79	76	87	81 ± 3.2
S. sanguis 7863	3	3	2	3 ± 0.3

Table 6.4 Hydrophobicity of bacteria suspended in PBS plus saliva.

Bacterium	Percentage reduction in absorbance of aqueous phase after mixing with xylene			
	experiment number			
	1	2	3	mean ± SEM
B. gingivalis P4	86	83	84	84 ± 0.9
B. gingivalis W83	70	68	75	71 ± 2.1
B. intermedius P2	43	62	55	53 ± 5.5
B. intermedius 9336	45	31	36	37 ± 4.1
Capnocytophaga sp. P2	57	56	63	59 ± 2.2
Capnocytophaga sp. 27872	61	43	39	48 ± 6.8
H. aphrophilus P5	63	57	61	60 ± 1.8
H. actinomycetemc. 9710	89	81	86	85 ± 2.3
Peptostrep. sp. P2	40	44	40	41 ± 1.3
Peptostrep. sp. 9807	14	8	11	11 ± 1.7
Veillonella sp. P3	94	91	98	94 ± 2.0
Veillonella sp. 11463	65	56	60	60 ± 2.6
A. israelii P2	36	28	36	33 ± 2.7
A. israelii 10215	96	96	97	96 ± 0.3
S. salivarius P2	39	40	40	40 ± 0.3
S. salivarius 8618	72	72	66	70 ± 2.0
S. sanguis P1	100	94	98	97 ± 1.8
S. sanguis 7863	17	13	20	17 ± 1.7

Table 6.5 Summary of bacterial hydrophobicity results in SIB, SIB plus saliva, PBS and PBS plus saliva.

Bacterium	Percentage reduction in absorbance of aqueous phase after mixing with xylene			
	SIB	SIB plus saliva	PBS	PBS plus saliva
B. gingivalis P4	97	96	70	84
B. gingivalis W83	75	67	60	71
B. intermedius P2	98	75	33	53
B. intermedius 9336	88	76	50	37
Capnocytophaga sp. P2	77	60	36	59
Capnocytophaga sp. 27872	88	86	60	48
H. aphrophilus P5	86	77	64	60
H. actinomycetemc. 9710	89	87	86	85
Peptostrep. sp. P2	99	44	65	41
Peptostrep. sp. 9807	100	57	61	11
Veillonella sp. P3	99	91	36	94
Veillonella sp. 11463	98	60	67	60
A. israelii P2	92	11	15	33
A. israelii 10215	86	73	3	96
S. salivarius P2	99	66	83	40
S. salivarius 8618	96	71	90	70
S. sanguis P1	99	96	81	97
S. sanguis 7863	62	0	3	17
MEAN	90	66	54	59

6.4 DISCUSSION

6.4.1 Experimental method

A variety of techniques have been used to assess bacterial hydrophobicity. Ciardi et al. (1983) studied the adherence of S. sanquis and S. salivarius to hydrophobic polystyrene and less hydrophobic glass. Gerson and Scheer (1980) investigated the adherence of several non-oral bacteria to five plastics with different hydrophobic properties. They concluded that the partitioning of bacteria between a liquid and a solid phase, and between two immiscible liquid phases, involved similar thermodynamic interactions.

Kjelleberg et al. (1980) determined the hydrophobicities of several non-oral bacteria according to their ability to bind a radiolabelled immiscible hydrocarbon, namely dodecanoic acid, a twelve carbon fatty acid. Dodecanoic acid was mixed with suspensions of bacteria and the amount absorbed was related to the hydrophobicity of the bacteria.

A standard biochemical technique for the separation of proteins from complex mixtures such as saliva or serum involves selective precipitation of the proteins with high concentrations of salts. Studies have shown that hydrophobic proteins precipitate at lower concentrations than hydrophilic proteins (Hjerten, 1981). Lindahl et al. (1981) applied this technique to bacterial cells, by using different concentrations of ammonium sulphate added to bacterial suspensions which then aggregated at different salt concentrations depending on the hydrophobicity of the bacteria. This method did of

course assume that bacterial cells behave in the same way as proteins, and did not take into account the possibility of specific bacterial aggregation.

Sherman et al. (1985) studied bacterial hydrophobicity with ammonium sulphate precipitation and compared this method with a hydrophobic interaction chromatography (HIC) method described by Stjernstrom et al. (1977). This latter technique used short columns prepared in pasteur pipettes plugged with glass wool and packed with phenyl-sepharose. Buffered suspensions of radiolabelled bacteria were added to the columns and eluted with buffer. The radioactivity levels of washes from the columns and of the sepharose gel were then determined. It was found that hydrophobic bacteria adhered to the gel imparting a high count. The results from this technique were reported to be comparable to those obtained with the ammonium sulphate precipitation method.

Olsson and Westergren (1982) compared the HIC method to the liquid hydrocarbon adherence assay of Rosenberg et al. (1980) used in this study. A number of inaccuracies in the HIC method were mentioned, for example, it was suggested that chains of streptococci may become mechanically trapped by the sepharose gel. In support of this, it was noted that the majority of the bacteria that had been retained by the sepharose were subsequently released if the gel was removed from the column and gently washed. In addition, some streptococci are known to bind to dextran (Mukasa and Slade, 1973) which is chemically relate to sepharose, and differences were noted between the retention of streptococci to ordinary sepharose compared with octyl-sepharose. Despite these problems, it was noted that a

generally good agreement existed between the two methods (Olsson and Westergren, 1982; Wilson et al., 1984). However, Rosenberg et al. (1981) presented several overriding advantages with the hexadecane assay: the hydrocarbon surface is well defined; elaborate equipment is not required; and quantitative results are rapidly and easily obtained.

The original hydrocarbon adherence method (Rosenberg et al., 1980) used three hydrocarbons, namely hexadecane, octane and xylene, and Nesbitt et al. (1982a) used hexadecane and toluene. Most researchers using this method have used hexadecane, although no reasons for this preference have been stated. Initially for this study, hexadecane and xylene were used, but with both of these methods poor reproducibility between repeated experiments was noted, similar to the results of some previous reports (Nesbitt et al., 1982a; Olsson and Westergren, 1982; Beighton, 1984). In addition, anomalous results were obtained with some of the strains tested. The absorbancies of some of the supensions after mixing with a hydrocarbon, were found to be higher than the initial absorbancies; for example, with the Capnocytophaga species up to twice the original turbidity was noted. These results were in contrast to the expected drop in absorbance with hydrophobic bacteria, or the unchanged absorbance with hydrophilic bacteria.

A close review of the data presented by other researchers revealed that similar anomalous results had been reported previously, but only with a small proportion of the strains tested. Nesbitt et al. (1982a) and Rosenberg et al. (1980) presented results giving

increases of up to 5 per cent, and Olsson and Westergren (1982) presented an increase with one strain of approximately 40 per cent. However, none of these workers discussed these aberrant results. Recently, Handley and Tipler (1986) noted similar increases, up to 8 per cent, with 6 out of the 10 Bacteroides species that they tested. The increases were attributed to the release of many small vesicles by the bacteria as a result of vortex mixing. This possibility may have been ruled out if separate controls had been prepared and compared with the hexadecane added test suspensions. Instead, the original absorbancies of the test suspensions were compared to the final absorbancies, as in the original method of Rosenberg et al. (1980). The use of separate controls, treated in the same way as the tests but without xylene, takes into account such possibilities as the aggregation or lysis of bacteria during incubation in the buffer or the release of vesicles due to vortex mixing. B. gingivalis W83 was one the strains suggested by Handley and Tipler (1986) to produce an increase in absorbance by the release of vesicles, however no evidence to support this was obtained in the present study with this strain or any other strain using separate controls.

An alternative explanation of this phenomenon is that residual particles of hydrocarbon adhere to the bacterial surface and increase the refractive index of the bacteria. If only a little hydrocarbon adheres, the bacteria may not be removed from the aqueous phase on separation of the phases. Consequently, the absorbance of the aqueous suspension will be increased because of the higher refractive index of the hydrocarbon coated cells.

If this hypothesis is true, then the removal of adsorbed hydrocarbon will cause a decrease in absorbance. Xylene, unlike hexadecane, is a volatile liquid, a property that can conveniently be used to remove it from an aqueous suspension. In the present study this was facilitated by bubbling air through the xylene-contaminated aqueous suspensions. Initially the absorbance of the suspensions was seen to fall rapidly and then levelled out after all the xylene had been removed (within two minutes).

The effect of this procedure with xylene on the strains that gave the largest increases (up to twice the original absorbance) was to reduce the absorbance to a level indicating that they were moderately hydrophobic, for example with the Capnocytophaga species (Table 6.5). This suggests that these bacteria are sufficiently hydrophobic to bind a limited amount of xylene, but not enough to ensure their removal from the aqueous phase.

In addition, it was noticed that vortex mixing of the aspirated aqueous phases also resulted in the liberation of xylene and could be used instead of the aeration procedure, but was less efficient. The effect of vortex mixing hexadecane-contaminated suspensions was to produce a wide range of absorbance values depending on the extent of mixing, probably due to the detachment and readsorption of hexadecane particles. This could also account for the poor reproducibility of repeated experiments using hexadecane noted in the previous reports of Rosenberg et al. (1980), Nesbitt et al. (1982a) and Olsson and Westergren (1982).

A possible criticism of the xylene method is that the reduction in absorbance noted with hydrophobic bacteria may be due, not to the removal of the bacterial cells into the hydrocarbon phase, but to the lysis of the cells by xylene. To investigate this possibility with hexadecane, Rosenberg et al. (1981) added isopropanol as a surfactant which resulted in the release of bacteria from the hexadecane layer and a recovery of most of the original absorbance. Nesbitt et al., (1982a) also considered that the hexadecane or toluene they used might disrupt the bacterial cell membrane. To ascertain if this occurred, they radiolabelled a strain of S. sanguis prior to performing the hydrophobicity assay. Both the aqueous and hydrocarbon phases were subsequently centrifuged to remove intact cells and were found to be free of radioactivity, thus indicating that the bacteria were not disrupted by the organic solvents. An alternative method was used in this study; the aqueous and xylene layers were aerated overnight to evaporate the xylene, returning the cells to the aqueous phase. This also recovered most of the original absorbance. Therefore, lysis would not appear to have a measurable effect on the results. However, even if cell lysis were pronounced, it would tend to affect only hydrophobic bacteria with which the hydrocarbon could interact. Hydrophilic bacteria would be protected by a water micelle preventing contact with the cell. Thus, if the bacteria tested were equally prone to lysis, this property should be comparable to their hydrophobic properties and would not be expected to markedly influence the hydrophobicity assay results. Therefore, the results of this study suggest that there are advantages in using xylene rather than hexadecane in hydrophobicity assays.

6.4.2 Bacterial hydrophobicity

Reference to Table 6.5 shows a wide variance between different strains and between the same strains tested in different suspending media. The variance evident between strains in this study was also noted between strains of the same species of oral streptococci by Westergren (1981), Olsson and Westergren (1982), Nesbitt et al. (1982a) and Wadstrom et al. (1984). However, contradicting results could be due to a number of reasons other than strain differences. Experimental variables such as the suspending medium used, the choice of hydrocarbon, vortex mixing times, incubation temperature, growth rate and medium and the age of the culture can all affect the results (Rosan et al., 1985). However, despite this variance the results of this study agree with a number of previous reports (Table 6.6). However, since much of the work on hydrophobicity has been done either with streptococci or non-oral organisms there are few reports in the literature with which to compare the bacterial species used in this study.

Most of the strains used here were predominantly hydrophobic, especially in SIB, which correlates with the reports of Weiss et al. (1982) and Rosenberg et al. (1983a) that the majority of oral bacteria are hydrophobic. However, the results of this investigation show clearly that the composition of the suspending medium can affect hydrophobicity. This was also shown by Rogers et al. (1984) who assessed the hydrophobicity of oral streptococci in three buffers, potassium/urea/magnesium (PUM) buffer, phosphate buffer and Hepes buffer. PUM buffer gave the highest hydrophobicity results compared with the low ionic strength phosphate buffer and zwitterionic Hepes

Table 6.6 Summary of bacterial hydrophobicity results from previous reports (references 1 to 5 are listed in Appendix 12) and from this study in SIB, saliva in SIB, PBS and saliva in PBS.

Bacterium	Bacterial hydrophobicity					
	Previous reports	Present study: strain	SIB	SIB plus saliva	PBS	PBS plus saliva
B. gingivalis	high[3] low[2]	P4	high	high	high	high
		W83	high	high	high	high
B. intermedius	high[2,3]	P2	high	high	low	high
		9336	high	high	high	low
Capnocytophaga sp.	ND*	P2	high	high	low	high
		27872	high	high	high	low
H. aphrophilus	low[1]	P5	high	high	high	high
H. actinomycetemc.	low[3]	9710	high	high	high	high
Peptostrep. sp.	ND	P2	high	low	high	low
		9807	high	high	high	low
Veillonella sp.	ND	P3	high	high	low	high
		11463	high	high	high	high
A. israelii	ND	P2	high	low	low	low
		10215	high	high	low	high
S. salivarius	high[4-6] low[3,7]	P2	high	high	high	low
		8618	high	high	high	high
S. sanguis	high[3-8]	P1	high	high	high	high
		7863	high	low	low	low

*ND = no data available.

buffer that gave the lowest hydrophobicity. It was suggested that the main factor affecting hydrophobicity was the ionic strength of the buffers which can enhance the partitioning of the bacteria between phases.

The differences in hydrophobicity noted with SIB or PBS may be due to the ionic concentrations of the buffers. Increased salt concentrations are known to cause the precipitation, or 'salting-out', of proteins or bacteria according to their degree of hydrophobicity (Lindahl et al., 1981). Therefore, it might be expected that PBS, with a higher ionic strength (Appendix 9) than SIB (Appendix 8), would produce higher bacterial hydrophobicity values. However, these relatively low ionic concentrations would not be expected to produce marked effects on hydrophobicity (Nesbitt et al., 1982a). Instead, 'salting-in' is more likely to occur, a phenomenon that has been widely demonstrated with proteins. Small concentrations of ions can cause slight changes in ionisation of amino-acid side chains and can also interfere with interactions between protein molecules by orientating between opposing charges, thus increasing protein solubility (Palmer, 1985). The results obtained in the present study suggest that 'salting-in' also occurs with bacteria. Thus, the ionic composition of PBS appears to provide a more favourable environment for the expression of the hydrophilic properties of bacteria.

Conversely, solutions with ionic concentrations similar to SIB, and hence saliva, may favour the expression of hydrophobicity. However, the addition of saliva to SIB resulted in hydrophobicity values that were, overall, significantly lower compared with SIB alone ($p < 0.01$), possibly due to salivary components coating the bacteria.

In support of this suggestion, Abbott and Hayes (1984) produced data indicating that adsorbed salivary components have a negatively charged hydrophilic nature. These components may therefore be important in mediating bacterial hydrophobicity *in vivo*. Beighton *et al.* (1984) also investigated the effect of saliva on hydrophobicity using oral streptococci isolated from macaque monkeys. They found either an increase or a decrease in measured hydrophobicity depending on the species or strains used. However, in these studies the bacteria were pretreated with saliva and the assays were carried out in experimental buffers. Bacteria *in vivo* will adsorb salivary components and will also be suspended in saliva. Therefore, of the suspending media used in this study, saliva plus SIB is the closest to the *in vivo* situation and the results suggest that *in vivo* approximately half of the bacteria tested will be only moderately hydrophobic and a few will be hydrophilic. This data disagrees with the conclusions of most other researchers that oral bacteria are predominantly hydrophobic. However, this is perhaps not surprising since none of these workers performed their assays in the presence of saliva.

6.4.3 Hydrophobicity, adherence and aggregation

A number of reports have associated adherence with hydrophobicity (Rosenberg *et al.*, 1983b), but there is also evidence to refute this suggestion (Rosan *et al.*, 1985) and consequently the relationship between these two phenomena is unclear. Most of the reports that have suggested this relationship have obtained data using two dissimilar buffers to measure hydrophobicity and adherence (Rosenberg *et al.*, 1981; Gibbons and Etherden, 1983). Both adherence

(Yamazaki et al., 1981; Eifert et al., 1984) and hydrophobicity (Rogers et al., 1984) have been shown to be affected by the composition of buffer used to suspend the test bacteria, which may affect the associations suggested.

In this study the hydrophobicity and adherence potential of a number of bacteria were assessed under similar experimental conditions. The adherence results (determined in SIB) from Chapters 2, 3 and 4 are summarized in Table 6.7 with the SIB hydrophobicity results from Table 6.5. Significant overall correlations were evident between hydrophobicity and adherence to buccal cells, saliva treated HeLa cells and saliva treated tooth enamel. The correlation coefficients of 0.536, 0.469 and 0.431, respectively, gave significance levels of 5, 5 and 10 per cent, respectively, with 16 degrees of freedom (18 pairs). However, no significant correlations ($p > 0.1$) were evident between hydrophobicity and adherence to SIB or serum treated HeLa cells.

The results also indicate that of the 11 strains that demonstrated high numbers of adhering bacteria (> 100 bacteria per 0.018 mm^2) to at least one surface, 9 were markedly hydrophobic (> 95 per cent decrease of absorbance of aqueous phase) and represented the 9 most hydrophobic bacteria tested. However, these bacteria did not adhere in high numbers to all of the surfaces tested, indicating that specific adherence interactions are required for bacteria to adhere in large numbers. In addition, less hydrophobic bacteria were not necessarily prevented from adhering in large numbers. These results therefore suggest that for bacteria to demonstrate a high affinity to a particular surface, specific interactions between the bacterial cell

Table 6.7 Bacterial adherence to buccal cells, SIB, saliva and serum treated HeLa cells and saliva treated tooth enamel (bacteria per 0.018 mm^2; Table 4.3) compared with bacterial hydrophobicity in SIB (percentage reduction; Table 6.5).

Bacterium	hydrophobicity in SIB	Bacterial adherence				
		buccal cells	HeLa cells			tooth enamel
			SIB	saliva	serum	
B. gingivalis P4	**97**	252	6	100	45	128
B. gingivalis W83	**75**	19	13	16	28	30
B. intermedius P2	**98**	38	1	8	1	336
B. intermedius 9336	**88**	8	0	0	0	7
Capnocytophaga sp. P2	**77**	30	3	4	1	79
Capnocytophaga sp. 27872	**88**	32	1	1	1	67
H. aphrophilus P5	**83**	68	6	15	23	24
H. actinomycetemc. 9710	**89**	64	19	93	100	324
Peptostrep. sp. P2	**99**	119	6	127	4	41
Peptostrep. sp. 9807	**100**	445	216	313	168	225
Veillonella sp. P3	**99**	709	120	139	268	584
Veillonella sp. 11463	**98**	176	29	88	5	151
A. israelii P2	**92**	14	2	16	3	24
A. israelii 10215	**86**	3	0	0	0	0
S. salivarius P2	**99**	309	17	397	4	47
S. salivarius 8618	**96**	22	26	325	6	22
S. sanguis P1	**99**	238	26	268	10	197
S. sanguis 7863	**62**	7	20	110	10	9
Significance*	--	$p<0.05$	NS+	$p<0.05$	NS	$p<0.1$

*Significance level of the correlation coefficient of a comparison with the hydrophobicity results.

+NS - not significant.

surface and the substrate surface must occur, which may be enhanced by, but does not require, hydrophobic structures on the bacterial cell surface. Hydrophobicity assays may therefore be used to identify bacterial isolates that are most likely to adhere well to oral surfaces.

Correlation coefficients were also calculated for different groups of bacteria, for example to determine if better correlations could be obtained with Gram-positive organisms, Gram-negative organisms, Gram-negative rods or streptococci. However, none of the groups tested gave more significant correlations than the whole group of 18 organisms.

Significant correlations were noted between hydrophobicity and adherence when both assays were performed in SIB. However, the use of different compositions of buffer resulted in a significant difference ($p < 0.01$) in the hydrophobicity results (Table 6.5) and consequently little correlation with adherence. Thus bacterial hydrophobicity and consequently bacterial adherence may be influenced by the composition of the suspending medium. This factor may partly account for the effect of buffer composition on adherence noted by other workers (Yamazaki *et al.*, 1981; Gibbons and Ethereden, 1983). Similarly, saliva added to SIB was shown to cause a significant ($p < 0.01$) decrease in hydrophobicity, and it has been reported that bacteria suspended in saliva adhere less well *in vitro* than when suspended in PBS (Slots and Gibbons, 1978). It may be of interest to determine if adherence experiments performed in different buffers or in the presence of saliva will correlate with hydrophobicity determined in the corresponding medium.

It has been reported that S. mutans may become less hydrophobic on repeated subculturing (Olsson and Westergren, 1982), but this was subsequently shown to occur with serotype c strains only (Westergren and Olsson, 1983). A consistent decrease in the hydrophobicity of the type strains compared with the fresh strains was not evident with the bacteria tested in this study. Therefore, unlike the adherence results obtained in the previous chapters (Table 6.7), hydrophobicity appears to be generally unaffected by laboratory maintenance of test strains, which suggests that the hydrophobic properties of bacterial cells are not necessarily mediated by their adherence conferring structures.

Homotypic aggregation was assayed in SIB, SIB plus saliva, PBS and PBS plus saliva, and the results were compared to the hydrophobicity values determined in the same corresponding media (Table 6.8). None of the correlations were statistically significant ($p > 0.1$). The results therefore suggest that hydrophobic interactions are not significant in mediating homotypic aggregation, including saliva induced aggregation.

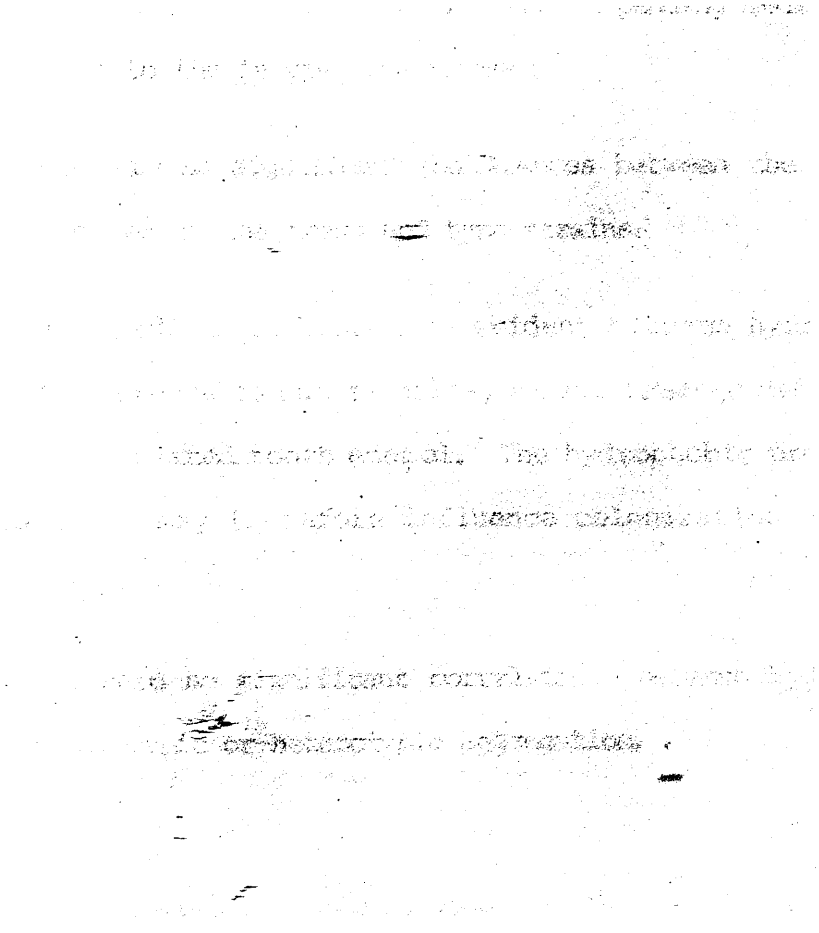
Heterotypic aggregation assays were performed in PBS with two different organisms. Therefore, aggregation may be influenced by the hydrophobic properties of both bacteria in PBS. Two possible interactions associated with bacterial hydrophobicity may occur; (i) aggregation between two bacteria may depend on the degree of hydrophobicity of the two organisms in aqueous suspension, in which case the sum of the hydrophobic properties of the bacteria may correlate with adherence, or (ii) aggregation between two bacteria may depend on the difference between their hydrophobic properties, ie.

Table 6.8 Comparison of homotypic aggregation ($t_{(Ao/2)}$ in minutes; Table 5.5) and hydrophobicity (percentage reduction; Table 6.5) results in SIB, SIB plus saliva, PBS and PBS plus saliva.

	aggregation:hydrophobicity			
Bacterium	SIB	SIB plus saliva	PBS	PBS plus saliva
B. gingivalis P4	166:97	292:96	371:70	246:84
B. gingivalis W83	636:75	878:67	616:60	1502:71
B. intermedius P2	249:98	184:75	163:33	126:53
B. intermedius 9336	493:88	626:76	340:50	606:37
Capnocytophaga sp. P2	540:77	32:60	239:36	65:59
Capnocytophaga sp. 27872	549:88	78:86	369:60	97:48
H. aphrophilus P5	24:83	32:77	108:64	45:60
H. actinomycetemc. 9710	61:89	111:87	355:86	931:85
Peptostrep. sp. P2	345:99	56:44	336:65	48:41
Peptostrep. sp. 9807	472:100	212:57	635:61	354:11
Veillonella sp. P3	22:99	56:91	135:36	145:94
Veillonella sp. 11463	214:98	36:60	367:67	26:60
A. israelii P2	281:92	257:11	325:15	274:33
A. israelii 10215	50:86	54:73	146:3	104:96
S. salivarius P2	43:99	39:66	273:83	88:40
S. salivarius 8618	453:96	39:71	395:90	35:70
S. sanguis P1	21:99	25:96	260:81	23:97
S. sanguis 7863	405:62	61:0	328:3	73:17
Correlation coefficient	-0.394	0.022	0.317	0.089
Significance	NS*	NS	NS	NS

*NS - not significant.

hydrophobic and hydrophilic bacteria may aggregate with each other, although theoretically this is less likely than (i). To determine if (i) occurred, the sum of the hydrophobicity values obtained for the 253 combinations of pairs of bacteria were calculated and compared with the corresponding 253 heterotypic aggregation results. For (ii), the differences between the hydrophobicity values were calculated. Both comparisons showed no significant correlations ($p > 0.1$), suggesting that hydrophobicity does not significantly affect heterotypic aggregation.



6.5 CONCLUSIONS

The method presented was rapid and simple to perform and may be used to screen bacterial isolates to select hydrophobic strains which are more likely to adhere well. The use of volatile hydrocarbons such as xylene are recommended for use with this assay to permit removal from the test suspensions of residual amounts of hydrocarbon, which may produce aberrant results.

The main points that arose from this study were:

(i) Approximately half of the bacteria studied were hydrophobic, half were moderately hydrophobic and a few were hydrophilic when tested in saliva plus SIB; the suspending media which was closest to the *in vivo* environment.

(ii) There were no significant differences between the hydrophobic properties of the fresh and type strains.

(iii) Significant correlations were evident between hydrophobicity and adherence to buccal cells, saliva treated HeLa cells and saliva treated tooth enamel. The hydrophobic properties of bacteria may therefore influence colonization of the oral cavity.

(iv) There were no significant correlations between hydrophobicity and homotypic or heterotypic aggregation.

CHAPTER 7

COLONIZATION OF THE GINGIVAL CREVICE BY BACTERIA IMPLICATED IN PERIODONTAL DISEASE

7.1 The gingival crevice area in health and disease

Different species of oral bacteria might be expected to attach only to specific types of surface. However the surfaces preferentially colonized by the bacteria implicated in periodontal disease have not been widely studied. The present study has investigated some of the factors involved in this process and may give an insight into the mechanisms involved in the colonization of the oral cavity by these bacteria. The aim of this chapter is to assemble the complex data from the previous chapters of the thesis, so that general conclusions can be drawn about the main factors involved in the colonization of the mouth, and particularly the gingival crevice area, by the 10 species of bacteria studied. It should be noted, however, that only one or two strains of each species were studied and large differences were noted in some cases between strains of the same species. Therefore, much more work will be required before a clear and conclusive picture will emerge.

The habitat of bacteria implicated in periodontal disease is primarily the gingival crevice. However, they can also be found in low numbers on the buccal mucosa, the dorsum of the tongue and the tonsils (van der Velden, et al 1986). These sites may serve as a reservoir from which bacteria can colonize the gingival crevice under suitable

conditions (van Winkelhoff et al., 1986). However, colonization is likely to depend on a complex number of variables, including the growth requirements of the bacteria, the numbers of organisms present and their ability to adhere to surfaces, as well as the disease state of the individual.

In relation to this latter factor, the healthy gingival crevice area (Figure 7.1a) usually presents a shallow gingival crevice with minimal crevicular fluid secretion and with small amounts of supragingival plaque predominated by Streptococci and Actinomyces species (Slots, 1977b). Therefore, the main surfaces available for colonization in the healthy gingival crevice region are likely to be saliva coated epithelial and enamel surfaces, and supragingival plaque consisting of mainly Gram-positive bacteria.

When gingivitis occurs, increased amounts of plaque are usually found and the gingivae become inflamed and swollen with deepening of the gingival crevice (Listgarten, 1976). The increase in plaque mass causes a reduction in the Eh of the gingival crevice area and crevicular fluid secretion increases (Kenney and Ash, 1969; van Palenstein Helderman, 1981a; Cimasoni, 1983). These conditions provide a more favourable environment for the colonization and proliferation of Gram-negative anaerobic and microaerophilic rods, actinomyces and fusiform bacilli (Slots et al., 1978). Therefore, while the surfaces available for colonization will include those present in the healthy gingival crevice, the altered plaque flora and the secretion of crevicular fluid may modify the environment and so affect colonization.

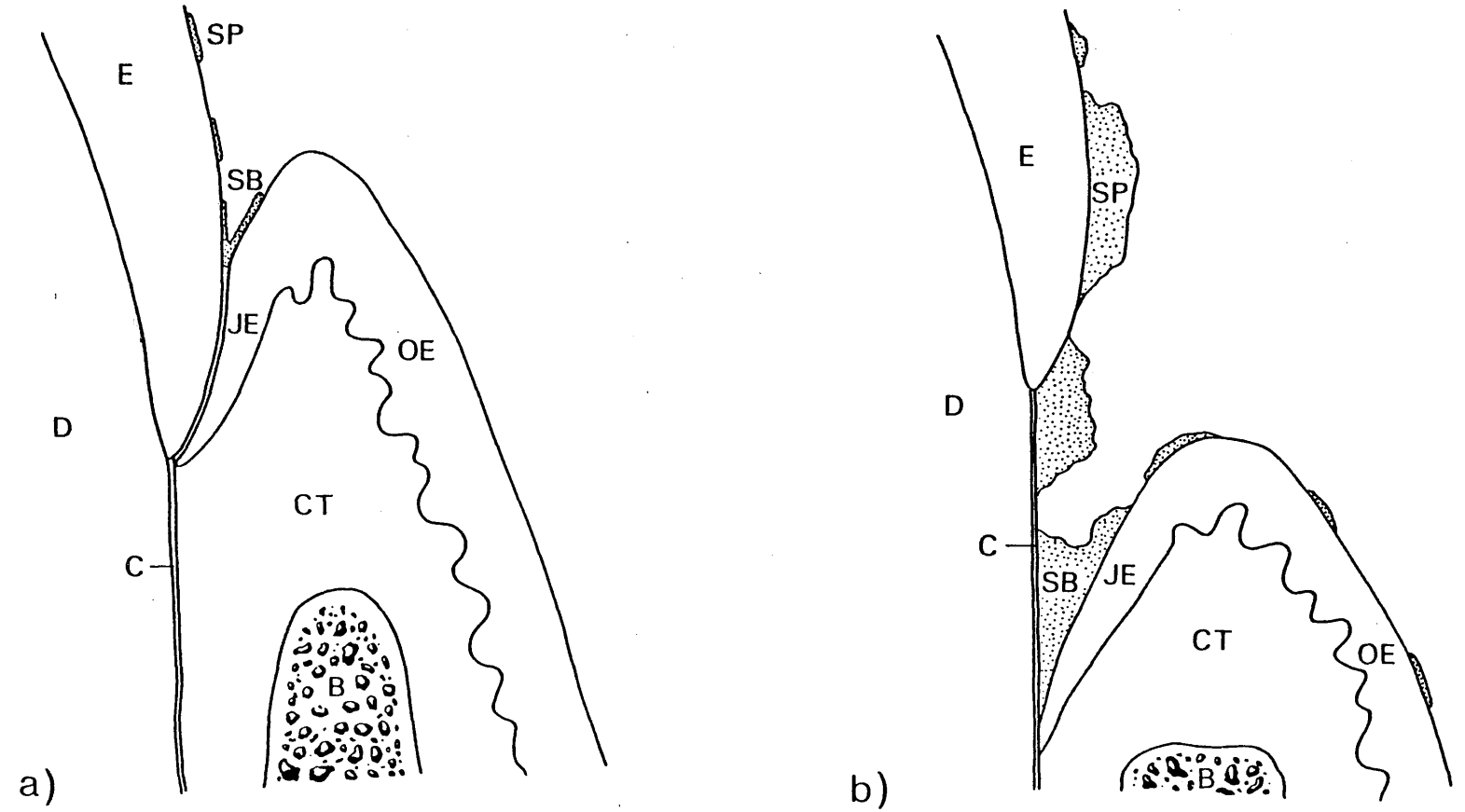


Figure 7.1 Diagrammatic representations of labial-lingual sections of the gingival crevice region; (a) healthy gingival sulcus and (b) sulcus in periodontal disease showing bone resorption, pocket formation and accumulations of subgingival plaque. C = cementum; CT = connective tissue; B = bone; D = dentine; E = enamel; JE = junctional epithelium; O = oral epithelium; SB = subgingival plaque; SP = supragingival plaque.

As the disease progresses, plaque accumulates, the gingival crevice deepens and the secretion of crevicular fluid increases further. This provides the anaerobic conditions and essential nutrients required by some of the bacteria associated with periodontal disease (van Palenstein Helderman, 1981a). As a result of these changes, the surfaces now available for colonization may include saliva and crevicular fluid coated epithelial cells, enamel and cementum, as well as supragingival and subgingival plaque predominated by Gram-positive and Gram-negative bacteria, respectively (Figure 7.1b).

7.2 Colonization by Bacteroides gingivalis

B. gingivalis is predominantly found in the gingival crevice and has been implicated in the aetiology of periodontal disease (Takazoe et al., 1984; Haffajee et al., 1986). The fresh and type strains of this organism demonstrated quite different properties with respect to their ability to adhere to the oral surfaces tested (Table 7.1). The fresh strain adhered in high numbers to buccal cells, saliva treated HeLa cells and saliva treated tooth enamel, but the type strain adhered in low numbers to all of the test surfaces. However, both strains adhered better to serum coated HeLa cells compared to SIB treated HeLa cells. In addition, the B. gingivalis strains were shown to aggregate heterotypically with six of the eight species against which they were tested, namely B. intermedius, Capnocytophaga species, Veillonella species, A. israelii, S. salivarius and S. sanguis.

B. gingivalis may therefore colonize by adhering to saliva coated epithelial and enamel surfaces and to supragingival plaques

Table 7.1 Summary of B. gingivalis adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
B. gingivalis P4	252	6	100	45	128	34	41
B. gingivalis W83	19	13	16	28	30	0	59

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

near the gingival crevice containing Streptococcus, Veillonella and Actinomyces species. It may also colonize by adhering to crevicular fluid coated epithelial cells within the gingival crevice and to subgingival plaque containing B. intermedius and Capnocytophaga species. In addition, heterotypic aggregation of B. gingivalis with other Gram-negative organisms within the periodontal pocket may assist the formation of stable subgingival plaque deposits which may aid their retention.

7.3 Colonization by Bacteroides intermedius

B. intermedius occupies a similar niche to B. gingivalis and likewise has been implicated in periodontal disease (Zambon et al., 1981; Slots, 1982b; Haffajee et al., 1986). Both strains of B. intermedius adhered poorly to the surfaces tested with the exception of the fresh strain which adhered well to saliva treated tooth enamel (Table 7.2). However, both strains of B. intermedius demonstrated widespread heterotypic aggregation, especially the fresh strain which aggregated with all of the bacterial species tested. This suggests that the ability of B. intermedius to adhere to host oral tissues is of minor significance, unlike B. gingivalis, and that the major factor governing colonization is the ability of B. intermedius to aggregate with other bacteria. This property may assist both the initial adherence and the subsequent formation of stable subgingival plaque deposits.

Table 7.2 Summary of B. intermedius adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
B. intermedius P2	38	1	8	1	336	23	88
B. intermedius 9336	8	0	0	0	7	0	41

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

7.4 Colonization by Capnocytophaga species

Capnocytophaga species are found in the gingival crevice and have been associated with several types of periodontal disease (Slots et al., 1978; Mashimo et al., 1983), although its aetiological role has been doubted by some workers (Socransky et al., 1986). The two Capnocytophaga strains studied demonstrated remarkably similar adherence and salivary aggregation properties (Table 7.3). They adhered in moderate numbers to saliva treated enamel and comparatively poorly to the remaining surfaces tested. As if to compensate for their poor adherence, both strains produced marked aggregation in saliva. Also, the type strain aggregated heterotypically with all four Bacteroides strains and with most of the Gram-positive commensal bacteria. Thus, the major factors governing the colonization of Capnocytophaga species appears to be saliva mediated homotypic aggregation and possibly heterotypic aggregation with supragingival and subgingival plaque bacteria.

7.5 Colonization by Haemophilus species

H. aphrophilus has been found in supragingival and subgingival plaque and has been associated with periodontal diseases in a few studies, although its aetiological role is not clear (Kilian and Schiott, 1975; Kilian et al., 1976; Moore et al., 1982a and 1983; Liljemark et al., 1984). The H. aphrophilus strain generally adhered comparatively poorly, but moderately well to buccal cells and it also demonstrated a four-fold greater affinity for serum than SIB treated HeLa cells (Table 7.4). In addition, this strain aggregated well in saliva. Therefore, in vivo H. aphrophilus may adhere to epithelial

Table 7.3 Summary of Capnocytophaga species adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
Capnocytophaga species P2	30	3	4	1	79	73	18
Capnocytophaga species 27872	32	1	1	1	67	74	59

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

Table 7.4 Summary of Haemophilus species adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
H. aprophilus P5	68	6	15	23	24	58	18
H. actinomycetemcomitans 9710	64	19	93	100	324	0	6

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

cells exposed to saliva or crevicular fluid, and may form stable plaques under the influence of saliva.

H. actinomycetemcomitans has been implicated in the aetiology of juvenile periodontitis and is principally found in the gingival crevice (Slots et al., 1980; Mashimo et al., 1983; Zambon et al., 1983; Slots and Genco, 1984; Mandell et al., 1986; Tempro et al., 1986). The type H. actinomycetemcomitans strain demonstrated little homotypic or heterotypic aggregation, however this strain adhered in high numbers to buccal cells, saliva and serum treated HeLa cells and saliva treated enamel (Table 7.4). This suggests that the colonization of H. actinomycetemcomitans would be mediated mainly by adherence to mucosal or tooth surfaces exposed to saliva or crevicular fluid.

7.6 Colonization by Peptostreptococcus species

Peptostreptococcus species have been isolated from plaque, the dorsum of the tongue and saliva, and have been found in increased numbers in subgingival plaque associated with periodontal disease (Gibbons et al., 1964b; Socransky and Manganiello, 1971; Slots et al., 1978; Moore et al., 1982a, 1982b and 1983). The type Peptostreptococcus strain adhered somewhat better than the fresh strain, but both adhered in comparatively high numbers to most of the surfaces tested (Table 7.5). Both strains also aggregated in saliva, but demonstrated little heterotypic aggregation.

Colonization by Peptostreptococcus species is therefore probably mediated by adherence to a variety of oral surfaces, with plaque

Table 7.5 Summary of Peptostreptococcus species adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
Peptostreptococcus sp. P2	119	6	127	4	41	86	6
Peptostreptococcus sp. 9807	445	216	313	168	225	44	18

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

formation enhanced by saliva induced aggregation. This range of properties may account for the common presence of Peptostreptococcus species in dental plaque and on the dorsum of the tongue in health (Gibbons et al., 1964b; Socransky and Manganiello, 1971) as well as in periodontal pockets (Slots, 1979; Moore et al., 1982a, 1982b and 1983). Most bacteria implicated in periodontal disease are found only in low numbers in the absence of periodontally diseased sites (Slots, 1979) and those tested in the present study, with the exception of the Peptostreptococcus species, generally adhered poorly.

7.7 Colonization by Veillonella species

Veillonella species are commonly found in the oral cavity, mainly in plaque and on the dorsum of the tongue (Gibbons and van Houte, 1975) and are not generally regarded as being implicated in the aetiology of periodontal disease. As with the Peptostreptococcus species, the Veillonella strains adhered in high numbers to most of the surfaces tested (Table 7.6). In addition, the type strain aggregated in saliva and the fresh strain aggregated heterotypically with both Gram-positive and Gram-negative species. Like the Peptostreptococcus species, Veillonella are also found commonly on various oral surfaces in both health and disease (Gibbons and van Houte, 1975; Slots, 1979) which may be related to their ability to adhere to a wide range of oral surfaces and to aggregate homotypically and heterotypically.

Table 7.6 Summary of Veillonella species adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
Veillonella species P3	709	120	139	268	584	0	71
Veillonella species 11463	176	29	88	5	151	93	24

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

7.8 Colonization by Actinomyces israelii

A. israelii is found mainly in dental plaque and also on the dorsum of the tongue and in saliva (Gibbons and van Houte, 1975; Holdeman et al., 1977). The role of A. israelii in periodontal disease is uncertain (Jordan, 1982). Despite the common presence of this organism in the oral cavity, the two strains tested in the present study adhered and aggregated poorly (Table 7.7), offering little explanation of their mechanisms of colonization. A possible explanation for these apparently abberent results may be the removal of cell aggregates from broths prior to performing experiments. This step in the assay procedures was to provide uniform test suspensions and was necessary only with the A. israelii strains.

7.9 Colonization by Streptococcus salivarius

S. salivarius is found mainly on oral mucosal surfaces and in saliva (Gibbons and van Houte, 1975). This correlates with the pattern of adherence noted with the test strains, ie. they adhered well to buccal cells and saliva treated HeLa cells, but poorly to saliva treated enamel (Table 7.8). Both strains also aggregated well in saliva, which may aid the formation of bacterial accumulations on the dorsum of the tongue.

7.10 Colonization by Streptococcus sanguis

S. sanguis is found in large proportions in dental plaque as well as on mucosal surfaces (Gibbons and van Houte, 1975). The fresh S. sanguis strain used in this study (Table 7.9) correspondingly

Table 7.7 Summary of A. israelii adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
A. israelii P2	14	2	16	3	24	16	12
A. israelii 10215	3	0	0	0	0	29	35

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

Table 7.8 Summary of *S. salivarius* adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
S. salivarius P2	309	17	397	4	47	68	18
S. salivarius 8618	22	26	325	6	22	91	24

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

Table 7.9 Summary of S. sanguis adherence to buccal cells (Table 2.4), SIB, saliva and serum treated HeLa cells (Table 3.4) and saliva treated tooth enamel (Table 4.1), and saliva induced homotypic aggregation (Table 5.5) and heterotypic aggregation (Table 5.11).

Bacterium	BACTERIAL ADHERENCE*					AGGREGATION	
	buccal cells	HeLa cells			tooth enamel	saliva induced[x]	heter-otypic[+]
		SIB	saliva	serum			
S. sanguis P1	238	26	268	10	197	91	41
S. sanguis 7863	7	20	110	10	9	78	24

*number of bacteria adhering per 0.018 mm^2 of surface.

[x]percentage reduction in PBS plus saliva compared to PBS alone.

[+]percentage of strains causing marked aggregation with the test strain (marked aggregation refers to > 20 per cent reduction of the $t_{(Ao/2)}$ in the test mixture compared to the theoretical control mixture).

adhered well to saliva treated tooth enamel as well as to buccal cells and saliva treated HeLa cells. The type strain appeared to have lost some adherence properties, although both strains aggregated in saliva. Thus, colonization of S. sanguis may be mediated by adherence to a variety of oral surfaces, and bacterial accumulations may form under the influence of saliva induced aggregation.

7.11 CONCLUSIONS

Different bacteria were shown to possess varying properties with regard to adherence, aggregation and hydrophobicity, all of which may affect colonization. It is therefore difficult to predict which of the colonization mechanisms investigated is the most important. However, the majority of the Gram-negative rods tested (the species most often implicated in periodontal disease), tended to adhere poorly to epithelial and tooth surfaces, but instead demonstrated marked homotypic or heterotypic aggregation.

The ability to adhere to some oral surfaces indicates that this property may be required by these Gram-negative bacilli, possibly to colonize sites other than the gingival crevice, albeit in low numbers. These organisms may act as a reservoir of bacteria which may colonize the gingival crevice if conditions become favourable for them to do so. Adherence may also be necessary for the initial colonization of the gingival crevice area when conditions are conducive to the growth of bacteria implicated in periodontal disease. Once within the protected environment of the gingival crevice, however, these adherence mechanisms may not be required and some may be lost by subsequent generations. Instead, aggregation may be more important in

assisting the formation of stable subgingival plaques which are more resistant to removal. Thus, conditions within the periodontal pocket may be selective for bacteria which do not expend valuable energy producing unnecessary adherence components. Hence the adherence properties of bacteria isolated from this site may depend on the number of generations a strain has been established in the gingival crevice where phenotypic alterations may occur. This ties in with the observation that type strains had impaired adherence functions, although aggregation was unaffected. A protective mechanism that prevents the loss of aggregating activity may therefore exist. Further, if the loss of unnecessary adherence components *in vivo* confers a selective advantage, the loss of adherence widely reported with the *in vitro* maintenance of cultures may be a natural occurrence.

The possession of homotypic or heterotypic aggregating properties by all but one of the bacteria implicated in periodontal disease indicates that this may be a common essential requirement, irrespective of adherence. Therefore, inhibiting aggregation may be useful in the control of periodontal disease. Unfortunately, the current literature mainly serves to illustrate the complexity of bacterial aggregation reactions and offers little hope of finding a useful aggregation-inhibitory factor. However, despite the heterogeneity of oral bacteria, common key surface components may be present that might be exploited to inhibit a wide spectrum of aggregation reactions.

APPENDICES

APPENDIX 1. Horse blood agar.

Blood agar base (Gibco)[1]	40 g
Defibrinated horse blood (Gibco)	50 ml
Distilled water	1000 ml

1. Dissolve base in distilled water at 100°C.
2. Adjust pH to 7.3 ± 0.2.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 50°C and add sterile defibrinated horse blood.
5. Pour 15 ml volumes into 90 mm sterile petri dishes (Sterilin)[2].

Blood agar base constituents (per 1000 ml):

Beef heart infusion	2 g
Peptone 220	10 g
Peptone 140	6 g
Yeast extract	2 g
Sodium chloride	5 g
Agar	15 g

[1]Gibco Europe Ltd., Paisley, Scotland.

[2]Sterilin, Teddington, Middlessex, England.

APPENDIX 2. Campylobacter agar.

Tryptic soy agar (Gibco)	40 g
Defibrinated horse blood (Gibco)	50 ml
Vitamin K/haemin solution (Gibco)	10 ml
Campylobacter antibiotic mix (Gibco)	1 ml
Distilled water	1000 ml

1. Dissolve base at 100°C in distilled water.
2. Autoclave at 121°C for 15 minutes.
3. Cool to 50°C and add sterile defibrinated horse blood, vitamin K/haemin solution, and campylobacter antibiotic mix.
4. Pour 15 ml volumes into 90 mm sterile petri dishes (Sterilin).

Vitamin K/haemin solution constituents:

Vitamin K	0.0005 g/ml
Haemin	0.0005 g/ml

Skirrow's campylobacter antibiotic mix constituents:

Vancomycin	0.010 g/ml
Trimethoprim	0.005 g/ml
Polymyxin B	2500 IU/ml

SKIRROW, M.B. (1977) British Medical Journal, 2 July, 9-11.

APPENDIX 3. Mitis-salivarius agar (Difco)[3].

Mitis-salivarius agar base	90 g
Potassium tellurite (0.1 per cent)	10 ml
Distilled water	1000 ml

1. Dissolve base in distilled water at 100°C.
2. Adjust pH to 7.3 ± 0.2.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 50°C and add potassium tellurite solution.
5. Pour 15 ml volumes into 90 mm sterile petri dishes (Sterilin).

Mitis-salivarius agar base constituents (per 1000 ml):

Tryptose	10 g
Proteose peptone	10 g
Dextrose	1 g
Saccharose	50 g
Dipotassium phosphate	4 g
Trypan blue	0.075 g
Crystal violet	0.0008 g
Agar	15 g

CHAPMAN, G.H. (1946) American Journal of Digestive Diseases, **13**, 105.

[3]Difco Laboratories, Surrey, England.

APPENDIX 4. Teepol agar.

Neutralized bacteriological peptone (Oxoid)[4]	24.4 g
Cysteine hydrochloride (Sigma)[5]	0.6 g
Yeast extract (Difco)	6 g
Potassium nitrate (BDH)[6]	1.2 g
Bromo-thymol blue (0.2 per cent) (BDH)	10 ml
Bacto-agar (Difco)	14.6 g
Sodium lactate (60 per cent) (Sigma)	12 ml
Teepol 610 (1 per cent) (BDH)	24 ml
Glucose (10 per cent) (BDH)	100 ml
Vitamin K/haemin solution (Gibco)	10 ml
Distilled water	1000 ml

1. Dissolve peptone, cysteine hydrochloride, yeast extract, potassium nitrate, bromo-thymol blue and agar in distilled water at 100°C.
2. Adjust pH to 7.4 ± 0.2.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 50°C and add glucose solution, Teepol, sodium lactate and vitamin K/haemin solution (all filter sterilized)
5. Pour 15 ml volumes into 90 mm sterile petri dishes (Sterilin).

MACFARLANE, T.W. (1977) Journal of Clinical Pathology, **30**, 191-192.

Vitamin K/haemin solution constituents:

Vitamin K	0.0005 g/ml
Haemin	0.0005 g/ml

[4]Oxoid Ltd., Basingstoke, England.

[5]Sigma Chemical company, Poole, Dorset, England.

[6]BDH Chemicals, Poole, Dorset, England.

APPENDIX 5. Tryptic soy-serum-bacitracin-vancomycin agar.

Tryptic soy agar (Gibco)	40 g
Yeast extract (Difco)	1 g
Horse serum (Gibco)	100 ml
Bacitracin (0.75 per cent) (Sigma)	10 ml
Vancomycin (0.05 per cent) (Sigma)	10 ml
Distilled water	1000 ml

1. Dissolve tryptic soy agar and yeast extract in distilled water at 100°C.
2. Adjust pH to 7.3 ± 0.2.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 50°C and add horse serum, bacitracin and vancomycin.
5. Pour 15 ml volumes into 90 mm sterile petri dishes (Sterilin).

Tryptic soy agar constituents:

Peptone 140	15 g
Peptone 110	5 g
Sodium chloride	5 g
Agar	15 g

SLOTS, J. (1982) Journal of Clinical Microbiology, 15, 606-609.

APPENDIX 6. Anaerobe blood broth supplemented (ABB) (Gibco).

ABB	32 g
Distilled water	1000 ml

1. Dissolve ABB in distilled water at room temperature.
2. Dispense 20 ml volumes into glass McCartney bottles.
3. Autoclave at 121°C for 15 minutes.

ABB constituents:

Tryptone	10 g
Beef extract	2 g
Liver extract	3 g
Yeast extract	5 g
Glucose	5 g
Sodium chloride	5 g
Vitamin K	0.005 g
Haemin	0.005 g
Cysteine hydrochloride	1 g
Dithiothreitol	0.1 g
Sodium bicarbonate	0.9 g

APPENDIX 7. Tryptic soy broth (TSB) (Gibco).

TSB	30 g
Distilled water	1000 ml

1. Dissolve base in distilled water at room temperature.
2. Dispense 20 ml volumes into glass McCartney bottles.
3. Autoclave at 121°C for 15 minutes.

TSB constituents:

Peptone 140	17 g
Peptone 110	3 g
Sodium chloride	5 g
Potassium phosphate	2.5 g
Dextrose	2.5 g

APPENDIX 8. Saliva ions buffer (SIB).

Final buffer concentration:

Potassium phosphate, pH 7.2	1 mM
Potassium chloride	50 mM
Magnesium chloride	0.1 mM
Calcium chloride	1 mM

To make SIB concentrate:

Dipotassium hydrogen orthophosphate (BDH)	4.355 g
Potassium dihydrogen orthophosphate (BDH)	3.402 g
Potassium chloride (BDH)	186.4 g
Magnesium chloride (BDH)	1.017 g
Distilled water	1000 ml

1. Dissolve potassium phosphate, potassium chloride and magnesium chloride in distilled water to make SIB concentrate.
2. Dispense into 100 ml volumes.
3. Autoclave at 121°C for 15 minutes.

To make calcium chloride concentrate (100 mM):

Calcium chloride (BDH)	5.55 g
Distilled water	500 ml

5. Dissolve calcium chloride in distilled water.
6. Autoclave at 121°C for 15 minutes.

To make final strength buffer:

7. Add 100 ml of SIB concentrate to 4850 ml of distilled water.
8. Dispense into 495 ml volumes.
9. Autoclave at 121°C for 15 minutes.
10. When cool, add 5 ml 100mM calcium chloride to 495 ml buffer.

APPENDIX 9. Phosphate buffered saline (PBS)

Final buffer concentration:

Potassium phosphate, pH 7.2	67 mM
Sodium chloride	150 mM

1. Dissolve buffer in distilled water.
2. Dispense into 500 ml volumes.
3. Autoclave at 121°C for 15 minutes.

Buffer constituents:

Dipotassium hydrogen orthophosphate (BDH)	5.84 g
Potassium dihydrogen orthophosphate (BDH)	4.56 g
Sodium chloride (BDH)	8.77 g
Distilled water	1000 ml

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5. Gibbons and van Houte, 1975.
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36. Eifert et al., 1984.

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5. Westergren and Olsson, 1983.
6. Wilson et al., 1984.
7. Nesbitt et al., 1982a.
8. Gibbons et al., 1983b.

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