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STUDIES ON THE METABOLISM AND FORMATION OF DENTAL PLAQUE

by

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To Cheryl,

A C K N O W L E D G E M E N T S

To Dr. I. Kleinberg for the time, effort and encouragement which he so generously bestowed upon me.

To Paul Averbach, Richard Zloty, Don Craw and Murray Vimy for their excellent technical assistance during parts of these studies.

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To the National Research Council of Canada and the Medical Research Council of Canada for granting me Fellowships during these studies.

ABSTRACT

The importance of dental plaque in dental caries and periodontal disease is well established. Despite extensive investigations, the basic processes underlying these disease processes are still unclear. The studies in this thesis, therefore, have examined several aspects of the metabolism and formation of dental plaque, which appear to be relevant to these two disease processes.

In the first study, conditions were established under which suspensions of plaque and sediment exhibit similar glycolytic activities and produce similar pH curves. Cell concentration proved to be an important variable. Plaque and sediment incubation mixtures at a cell concentration of 8.3 and 16.7 per cent (V/V), respectively, yielded generally similar pH-time curves in the presence of (i) salivary supernatant, (ii) glucose, (iii) urea, (iv) D(-) and L(+) lactic acid, (v) fluoride and (vi) a salivary fraction containing pH-rise factor. Also, with these cell concentrations and in the presence of salivary supernatant, the buffering capacity of the plaque and sediment suspensions were similar. However, in the absence of salivary supernatant, the plaque exhibited a slightly greater buffering capacity.

The findings in these experiments support the hypothesis that the acid-base metabolisms of the plaque and sediment are similar and substantiate the use of salivary sediment as a model for studying the metabolism of dental plaque.

In the next study, the concentration of ammonia and urea in three day fasting plaques on the labial and interproximal surfaces of the maxillary and mandibular incisors were determined. The ammonia

levels showed the same pattern as the pH levels characteristic for these sites. That is, lowest in maxillary labial plaques, highest in mandibular interproximal plaques and intermediate levels in maxillary interproximal and mandibular labial plaques. In contrast, the urea levels were much lower, but at a similar concentration at all sites. Titration experiments showed that the buffering capacities of maxillary labial and mandibular interproximal plaques was similar and that the difference in pH between these plaques could be attributed to their difference in ammonia concentration.

These results support the hypothesis that plaque ammonia production is responsible for the pH differences at the various sites. The finding that the plaques with greater exposure to salivary urea have the same urea levels as plaques which do not can be accounted for by an increased ureolytic activity.

In the third study, aspects of the acid-base production diffusion theories were examined. Subjects who had not brushed their teeth for 3 days and had not eaten for at least the previous 12-hour period rinsed with a solution of either urea or glucose (0.28M). Following the rinse, the concentration of that substance was measured in the maxillary labial incisor plaques and the saliva at regular intervals for 30 min. Following the urea rinse, plaque ammonia concentrations were also measured.

The uptake of both substrates by the plaque was rapid and the clearance of each occurred exponentially. However, both the entry and the clearance processes were faster for urea than for glucose. In contrast, the disappearance of glucose from saliva was faster than that

of urea. During the urea rinse, ammonia accummulated in the plaque at the same rate as the urea was cleared. The clearance of ammonia from the plaque occurred at a much slower rate. By calculation it appeared that only 16-26 per cent of the ammonia that could have been formed was actually produced.

These findings support the contention that diffusion is a rate limiting step in the uptake of these substrates by the plaque. Further support is also added to the hypothesis that fasting plaque has a floc-like structure.

In the next study, the composition of the free amino acid pool in 3 day fasting plaques from different sites on the incisor teeth was determined and the effect of rinsing with urea and/or glucose on the composition of this pool was examined.

It was evident from this study that: (i) there are only small differences in the composition and size of the free amino acid pool in plaques from the different incisor sites; (ii) the composition of the plaque free amino acid pool is clearly different from that of hydrolysates of plaque matrix or the plaque bacteria; (iii) changes in the composition of the plaque pool can occur when the plaques are exposed to glucose and urea and these changes are confined chiefly to aspartic and glutamic acids and alanine; and (iv) a large increase in alanine in the plaque free amino acid pool is dependent on the presence of both glucose and urea.

These experiments substantiate that urea and glucose are catabolized in a similar manner in plaque <u>in situ</u> and in salivary sediment system and suggest an important role for alanine in the acid-base

metabolism of the dental plaque.

In the final study, the aggregation behaviour of suspensions of pure and binary mixtures of various oral streptococci and the mixed populations of plaque bacteria was examined by titration between pH 10 to 2.

On the basis of the aggregation patterns and the pH at which maximum aggregation occurred (MA pH), the 11 strains of streptococci examined could be classified into 3 types. The MA pH was highest for the Type I organisms, less for the Type II and least for the Type III. Increasing the ionic strength or adding calcium to the suspending medium resulted in a shift of the MA pH to higher values. This effect was small for Strep. mutans strains AHT, BHT, 6715, OMZ 176 and GS-5, Strep. sanguis 10556 and Strep. salivarius, but large for Strep. mutans strains PS-14 and Ingbritt and Strep. sanguis strains 10557 and 10558.

Continuous particle electrophoresis of the mixed population of cells obtained directly from dental plaque showed that differences in the surface of the bacteria naturally occurring dental plaque also exists.

The aggregation pattern of binary mixtures of streptococci was most like the organism within the mixture having the higher MA pH.

This behaviour, together with the results with the pure suspensions, indicates that an electrostatic mechanism is probably involved. Mixtures containing plaque microorganisms cultured from the tooth and the gingival margins or obtained directly from plaque behaved in a manner similar to the binary mixtures suggesting that similar processes are also involved in these cases.

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CHAPTER I

INTRODUCTION

It is well established that dental plaque plays an important role in both caries and periodontal disease, two of the most prevalent diseases in man. Although research into the nature of plaque formation and metabolism has increased markedly in the past several decades, many of the basic processes involved are not fully understood. This situation is due, in part, to the many technical obstacles which must be overcome in order to examine plaque *in situ*. For this reason several model systems have been developed (e.g. pure culture *in vitro*, salivary sediment system, animal model, clinical investigation).

The present chapter describes some of the available literature concerning the composition, metabolism, and formation of dental plaque which is relevent to these processes and the studies of this thesis.

Composition and Structure of Dental Plaque

The hard and soft tissues of the oral cavity harbour an indigenous microbial flora as do other body surfaces in close proximity to the external environment (ROSEBURY, 1962). In the mouth, these adherent bacterial masses are termed dental plaque (WILLIAMS, 1897; BLACK, 1898).

Dental plaque consists of both cellular and acellular components; the relative proportions are shown in Fig. 1.1 (from SILVERMAN and KLEINBERG, 1967). In terms of nitrogen, the cellular fraction constituted

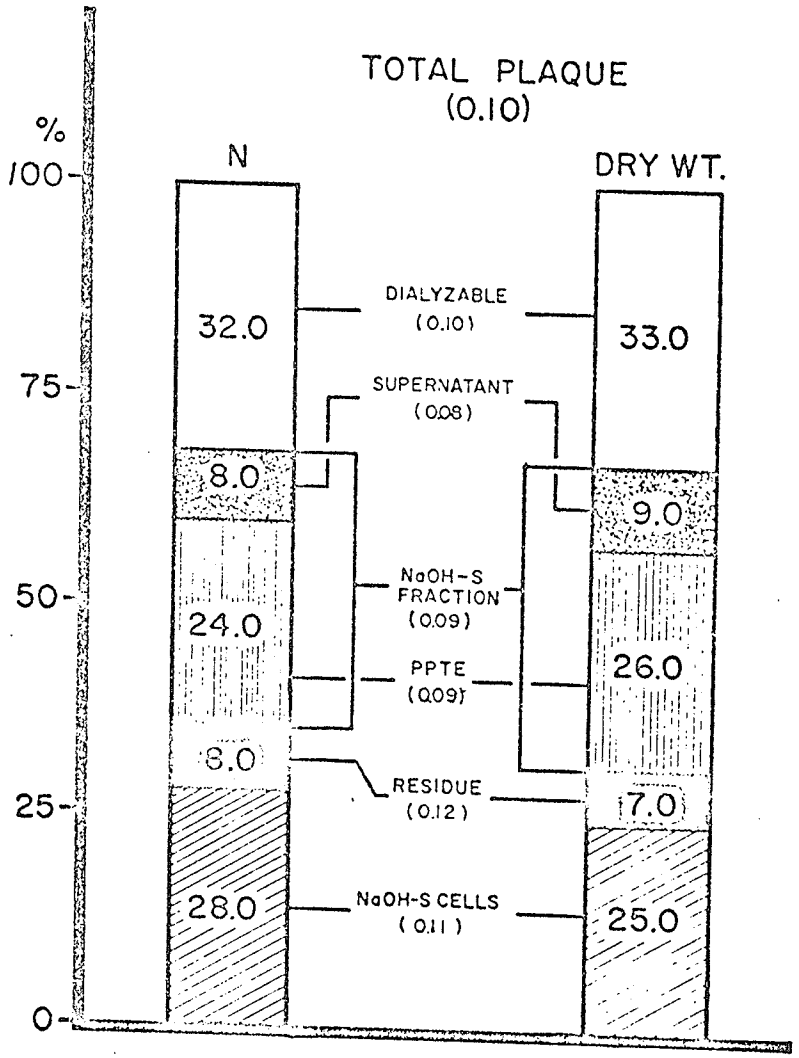


Fig. 1.1. Percentage composition of total plaque in terms of nitrogen (N)

about 36 percent of the total plaque, the acellular large molecular weight components about 32 percent, and the remaining small molecular weight constituents, most of which are probably of intracellular origin, constituted the remainder.

(a) Cellular composition of the supragingival plaque

One important characteristic of plaque is its very high cell density. On a numbers to weight basis, the total microorganisms in plaque approach that of a culture sediment or a bacterial colony grown on agar (STRALFORS, 1950).

The diversity of bacterial species found in plaque (HEMMENS et al., 1946), the difficulties in devising appropriate sampling techniques and growth media (CARLSSON, 1971 b; MANGANIELLO et al., 1971) the influence of dentition location (ONISI et al., 1957; IKEDA and SANDHAM, 1971), the variation in the diet (CARLSSON, 1967; FOLKE et al., 1972) and the duration of plaque accumulation (MANDEL et al., 1957; RITZ, 1967) are variables which make a comprehensive and reliable estimate of the microbial composition of plaque difficult to achieve. However, the use of rich non-selective media has yielded a general appreciation of the predominant microorganisms to be found in plaque.

At least 27 different types of organisms have been identified in plaque samples (HEMMENS et al., 1946). Those most frequently isolated were streptococci and diphtheroids. STRALFORS (1950) found in anaerobically cultured plaques from children the most predominant microorganisms to be streptococci (35 million per mg of plaque) and then members of the species Neisseria (10 million per mg of plaque). GIBBONS et al., (1964), also using anaerobic culture techniques, found a more varied plaque flora than STRALFORS but less so than HEMMENS et al. The proportion of bacterial

types amongst the organisms cultivable on heart infusion blood agar plates supplemented with 0.5 mg/ml menadione was: streptococci, approximately 28 percent; aerobic "diphtheroid" organisma, 24 percent; gram positive anaerobic rods, 18 percent; anaerobiv gram positive streptococci (Peptostreptococcus), 13 percent; and members of the species Neisseria, 3 percent.

(i) influence of duration of accumulation

Plaque, at least in its early stages of formation, changes in bacterial composition with time (MANDEL et al., 1957). Light microscopic examination of early plaque (1-2 days) reveals chiefly (90 percent) gram-positive and gram-negative cocci with a few gram-positive and gram-negative rods (THEILADE and THEILADE, 1969). Such early plaques contain fewer dead and degenerating cells than older plaques. During the next few days filamentous forms and slender rods predominate, though cocci are still present in large numbers. By twelve days, plaque appears to be composed almost entirely of irregular twisted strands of filamentous bacteria (MANDEL et al., 1957). With the electron microscope, it is possible to see at this stage some gram-positive and gram-negative cocci either arranged in distinct colony-like cell clusters or as a diffuse mixed cell population (SCHROEDER, 1970).

During bacteriologic examination, generally the same types of changes are apparent (RITZ, 1967). Streptococci were the predominant microorganisms averaging from 30 to 70 percent of the cultivable microflora during the first 9 days; the highest counts were at day three. Neisseria declined from an average of 9 percent at day one to 3 percent at day nine; similarly, Nocardia, initially comprising 6 percent of the total population, was only 0.1 percent by the ninth

day. On the other hand, the fusobacteria increased from 0.2 to 1 percent, Veillonella from 1.5 to 12 percent and Actinomyces from 3 to 23 percent.

(ii) influence of location

The plaque population can vary with dentition site. ONISI et al., (1957) found that approximal plaques from lower incisors and upper molars had a higher incidence of ureolytic microorganisms than plaques from other approximal sites. In children, Strep. mutans was found mainly in pits and fissures (39 percent of the total cultivable streptococci), infrequently on buccal surfaces (2.9 percent) and none could be detected on the approximal surfaces of lower molars (IKEDA and SANDHAM, 1971). Much higher levels of lactobacilli are found in pits and fissures, approximal surfaces and within carious lesion than in other locations in the human dentition (BAHN and QUILIMAN, 1963).

The predominant cultivable organisms in different sites of the adult oral cavity as compiled from various studies by SOCRANSKY and MANGANIELLO (1971) are shown in Table I.1. This is pooled data and therefore does not reflect the degree of variability that actually exists. It does show, however, the complexity of the microbiota that exists at all sites and that differences can exist amongst sites.

(iii) influence of diet

Although plaque will form on the teeth of tube-fed persons, such plaques are low in total streptococcal counts and have a much lower incidence of lactobacilli and filamentous organisms than plaques of normally fed persons (LITTLETON et al., 1967). Reduction of oral lactobacillus counts may also be induced through a reduction of dietary carbohydrate intake (BECKS et al., 1944; JAY, 1947; APPLETON, 1950). Whereas the incidence of Strep. sanguis increases in response

TABLE I.1. Mean Percentages of Cultivable Organisms in the Adult Oral Cavity*

	Gingival Crevice area	Dental Plaque	Tongue	Saliva
Gram Positive Facultative Cocci	28.8	28.2	44.8	46.2
Streptococci	27.1	27.9	38.3	41.0
S. salivarius	N.D.	N.D.	8.2	4.6
Enterococci	7.2		N.D.	1.3
Staphylococci	1.7	0.3	6.5	4.0
Gram Positive Anaerobic Cocci	7.4	12.6	4.2	13.0
Gram Negative Facultative Cocci	0.4	0.4	3.4	1.2
Gram Negative Anaerobic Cocci	10.7	6.4	16.0	15.9
Gram Positive Facultative Rods	15.3	23.8	13.0	11.8
Gram Positive Anaerobic Rods	20.2	18.4	8.2	4.8
Gram Negative Facultative Rods	1.2	N.D.	3.2	2.3
Gram Negative Anaerobic Rods	16.1	10.4	8.2	4.8
Fusobacterium	1.9	4.1	0.7	0.3
B. melaninogenicus	4.7	N.D	0.2	N.D.
V. sputorum	3.8	1.3	2.2	2.1
other Bacteroides	5.6	4.8	5.1	2.4
Spirochetes	1.0	N.D.	N.D.	N.D.

N.D. = not detected.

*(From, Socransky and Manganiello, 1971).

to a reduced intake of dietary carbohydrate, the incidence of Strep. mutans is reduced (de STOPPELAAR et al., 1970).

The type of dietary carbohydrate may also influence the composition of the oral microflora. For instance, Strep. salivarius in both saliva and dental plaque increased to a greater extent when sucrose replaced glucose in the diet (CARLSSON and SUNDSTROM, 1968).

The microflora may be affected by other nutritional factors since rats fed a high protein diet were found to harbour more than twice the number of gram-positive, facultative, pleomorphic rods than their litter mates fed on a Purina lab chow diet (SOCRANSKY et al., 1969).

(iv) iatrogenic influences

In an effort to treat disease, alterations of the oral environment may occur which result in changes in the plaque bacterial population. BALENSEIFEN and MADONIA (1970) found a large increase in the incidence of Strep. salivarius, Strep. mitis and members of the species Lactobacillus in plaques four to five weeks after orthodontic bands were placed.

In the presence of palatal acrylic plates which simulate partial dentures (ONISI and KONDO, 1956), or partial dentures themselves (GUSTAVSON, 1964), the Lactobacillus counts in plaque tend to increase.

Irradiation of the main salivary glands for cancer treatment may be followed by atrophy of these glands and rampant caries. LLORY et al., (1971, 1972) noted several changes in the composition of the plaque microflora following such treatment. Examination of the aerobic flora showed an increase in the total number of streptococci and an increase in the proportions of Strep. mitis and members of the species Lactobacillus. On the other hand, the proportion of the corynebacteria

and the Neisseria decreased. Of the facultative plaque flora, a large increase in the proportions of Strep. mutans and yeasts was noted. The proportion of Strep. mutans increased from 0.6 to 43.8 percent of the facultative streptococci while the yeasts (chiefly Candida albicans) increased from a trace to 0.04 percent. The anaerobic microflora also showed major alterations in composition. Though the total number of strict anaerobes was stable, Actinomyces increased and Veillonella decreased. All of the changes noted persisted up to four years after irradiation. It was suggested that these changes are due to atrophy of the salivary glands rather than a direct effect of irradiation on the plaque bacteria since irradiation for treatment of carcinoma of the maxillary sinus did not produce such alterations.

(b) Acellular constituents of supragingival plaque

Besides low molecular weight products of bacterial metabolism and the inorganic salts derived from the saliva or the tooth, the major non-bacterial plaque component is an extracellular matrix within which the bacteria are embedded. This organic material consists of large molecular weight polysaccharides of bacterial origin (CARLSSON and EGELBERG, 1965; CRITCHLEY et al., 1967; GIBBONS and BANGHART, 1967; GUGGENHEIM and SCHROEDER, 1967) and glycoproteins derived from saliva (DOBBS, 1932; MANDEL, 1966; SILVERMAN and KLEINBERG, 1967).

(i) extracellular polysaccharides

After a sucrose rinse, plaques in vivo show an increase in the carbohydrate component of the matrix (CRITCHLEY et al., 1967). The major extracellular polysaccharides extractable from such plaques are glucans and fructans (McDOUGALL, 1964; CRITCHLEY et al., 1967). These are polymers of α-D-glucopyranose and D-fructo-furanose residues respectively.

The glucans are linked mainly by α-1,6 and α-1,3 linkages. Where the α-1,6 type of linkages are predominant, the polymer has been referred to as a dextran. Where the α-1,3 linkages are in high proportion, the polymer is more insoluble and has been referred to as a mutan (GUGGENHEIM, 1970). Fructans are linked mainly by β-2,6 linkages.

The formation of glucose and fructose polymers by the mixed oral flora is dependent upon both the amount of available sucrose and the environmental pH (HALHOUL and KLEINBERG, 1970). Formation is favoured by an increase in sucrose concentration but retarded as the environmental pH decreases below neutrality.

Until recently, most investigators believed that only levans were degradable by the plaque bacteria. This view had been based on the following findings: (i) plaque or bacterial isolates from saliva do not utilize dextran but do utilize levan (GIBBONS and BANGHART, 1967), (ii) animals or humans on a high sucrose supplemented diet (CARLSSON and EGELBERG, 1965; GIBBONS et al., 1966) accumulate large amounts of dextran in their plaques, and (iii) on analysis, dental plaque in situ contains much lower levels of levan than dextran (CRITCHLEY et al., 1967; WOOD, 1967). However, GOLD, et al., (1969) and HALHOUL and KLEINBERG (1972) have demonstrated that the mixed microflora of plaque and salivary sediment can degrade both dextrans and levans. Sucrose, by virtue of the glucose and fructose produced upon its hydrolysis, inhibits the catabolism of these polymers by the mixed oral flora (HALHOUL and KLEINBERG, 1972). Thus, in the studies where sucrose is more or less continuously present at high concentration, conditions would be favorable for the synthesis of extracellular polysaccharides, but not for their degradation. Dextrans and levans, therefore, tend to accumulate.

One fact which earlier investigators had not considered was the possibility that loss of more levan than dextran from plaque might contribute to the lower plaque levan than dextran levels. In this regard, CRITCHLEY et al., (1967) had the subjects of their study rinse twice with distilled water after the original sucrose rinse. This might have washed out more levan than dextran (HALHOUL, 1972).

(ii) glycoproteins

After thorough cleaning of the tooth surface, an amorphous acellular deposit rapidly forms; this material appears to be composed primarily of salivary glycoproteins (MECKEL, 1965; ARMSTRONG, 1967).

Electron microscopic observation of newly formed plaques in conjunction with carbohydrate and protein staining experiments have revealed that plaque matrix consists of a fibrous network of protein (CRITCHLEY et al., 1968) having an amino acid composition similar to glycoproteins, but with many of the sugar residues missing (LEACH, 1964; MIDDLETON, 1964; SILVERMAN and KLEINBERG, 1967).

When the supernatant of saliva is cleared of its cellular elements and dizlyzed against distilled water, a visible precipitate forms (KLEINBERG et al., 1971). This precipitate, which may appear between pH 6.5 and 3.2, contains calcium, phosphorus, carbohydrate and protein. X-ray diffraction analysis of the precipitate and of early plaques indicated that the salivary calcium phosphate carbohydrate-protein complex is present in both of these (KAUFMAN and KLEINBERG, 1973).

From a consideration of the above studies and those of McDOUGALL (1963) and SILVERMAN and KLEINBERG (1967) it appears that glycoprotein-like material derived from saliva may exist (1) as a condensed amorphous layer at the enamel interface of plaque, and (2) as a porous fibrous network constituting a portion of the extracellular

matrix of formed plaques (KLEINBERG, 1970 b).

(c) Structure and permeability properties of plaque

STRALFORS (1950), described the diffusion properties of plaque in terms of a gel structure and was able to demonstrate that bacteria incorporated within an agar gel could mimic the pH response of plaque *in situ* when exposed to glucose. On the basis of these experiments, STRALFORS (1950) proposed his acid production diffusion (APD) theory which states - that the accumulation of acid within plaque occurs when the rate of formation of acid by the plaque bacteria is more rapid than the rate at which acid can diffuse away into the saliva. Mathematically, the APD theory is described by the following equation:

$$U_o = \frac{QH^2}{2D_L}$$

where U_o is the acid concentration at the surface of the enamel, Q is the rate of acid production of the bacteria, H is the thickness of the plaque and D_L is the diffusion co-efficient for the acid in the plaque.

As pointed out by KLEINBERG (1970 b), however, a gel-like structure which limits the diffusion of metabolites out of the plaque would also retard the entry of substrate. As a result of a slowed entry of sugar into the plaque, the rapid accumulation of acid as demanded by the APD theory would not occur. On the basis of studies investigating the chemical forces responsible for plaque integrity (SILVERMAN and KLEINBERG, 1967 a and b) and studies showing rapid uptake of urea by fasting plaque *in situ* following a urea solution rinse (SINGER and KLEINBERG, 1969), KLEINBERG (1970 a and b) concluded that the basic structure of plaque may be a floc. The high porosity that floc structures can have (LaMER and HEALY, 1963), while allowing for easy

entry of substrate, may not allow for similar easy exit of the end-products of bacterial degradation since exit of fluids from narrow spaces is difficult (NEVIN, 1954).

Since the intercellular spaces of plaque can become filled with extracellular polysaccharide following extended exposure of the plaque bacteria to sucrose, the basic plaque structure of the floc may be modified to a more gel-like structure. Such alterations would change the permeability and diffusion properties of the plaque and both the rates of substrate entry and end-product exit.

Metabolism of the mixed oral flora

(a) Carbohydrate

When glucose is metabolized by plaque *in situ* or *in vitro* significant quantities of lactic, acetic and propionic acids are formed (MUNTZ, 1943; MOORE *et al.*, 1956; ENGLANDER *et al.*, 1959; GILMOUR and POOLE, 1967). Butyric, isobutyric and valeric acids have also been found but generally in much smaller quantities (RANKE *et al.*, 1963; GUGGENHEIM *et al.*, 1965).

The relationship between the major organic acids formed and the decrease in pH that occurs during the utilization of glucose by the mixed oral bacteria was examined using the suspended salivary sediment (SSS) system (KLEINBERG, 1967). At low glucose concentrations, as the pH rapidly fell, the lactic acid concentration rapidly increased; when the pH subsequently slowly rose, the lactic acid concentration slowly fell (SANDHAM and KLEINBERG, 1970 b). Although the onset of both the pH rise and the lactic acid decrease were dependent upon the glucose being used up, the lactic acid decrease began a little earlier than the pH rise. The other acids, mainly a mixture of propionic and acetic,

increased asymptotically. At high glucose concentration, where the pH falls to a lower level and does not show a subsequent rise, the lactic acid concentration rises to a more or less constant level early in the incubation period while the other acids continue their progressive rise. These results, together with those of MUNTZ (1943) and GEDDES (1972) with plaque, indicated that during the metabolism of glucose by the mixed oral flora, lactic acid is an intermediate while acetic and propionic acids are end-products of carbohydrate catabolism by the mixed bacterial populations of salivary sediment and dental plaque.

Using the SSS system and the technique of radiorespirometry, SANDHAM and KLEINBERG (1970 a) showed that most of the carbon dioxide produced from labelled glucose was derived from carbons-3 and -4 (94-97%), while only a small amount (3-6%) came from carbon-1. None was formed from glucose carbons-2 and -6. These investigators concluded that the major route of glucose metabolism was via the Embden-Meyerhof pathway of glycolysis; a minor amount was catabolized via the hexose monophosphate pathway, while almost none was consumed by the tricarboxylic acid cycle. On the basis of this study and that described above, SANDHAM and KLEINBERG (1970 b) proposed the scheme shown in Fig. 1.2 to account for the metabolism of glucose.

(b) Urea

(i) in vitro studies

Utilization of urea with concomitant ammonia production has been demonstrated for the mixed bacterial populations of whole saliva (CARY, 1946; BALLANTYNE et al., 1951) salivary sediment (BISWAS and KLEINBERG, 1971) and dental plaque (FROSTELL, 1960).

With regard to plaque, FROSTELL (1960), in agreement with

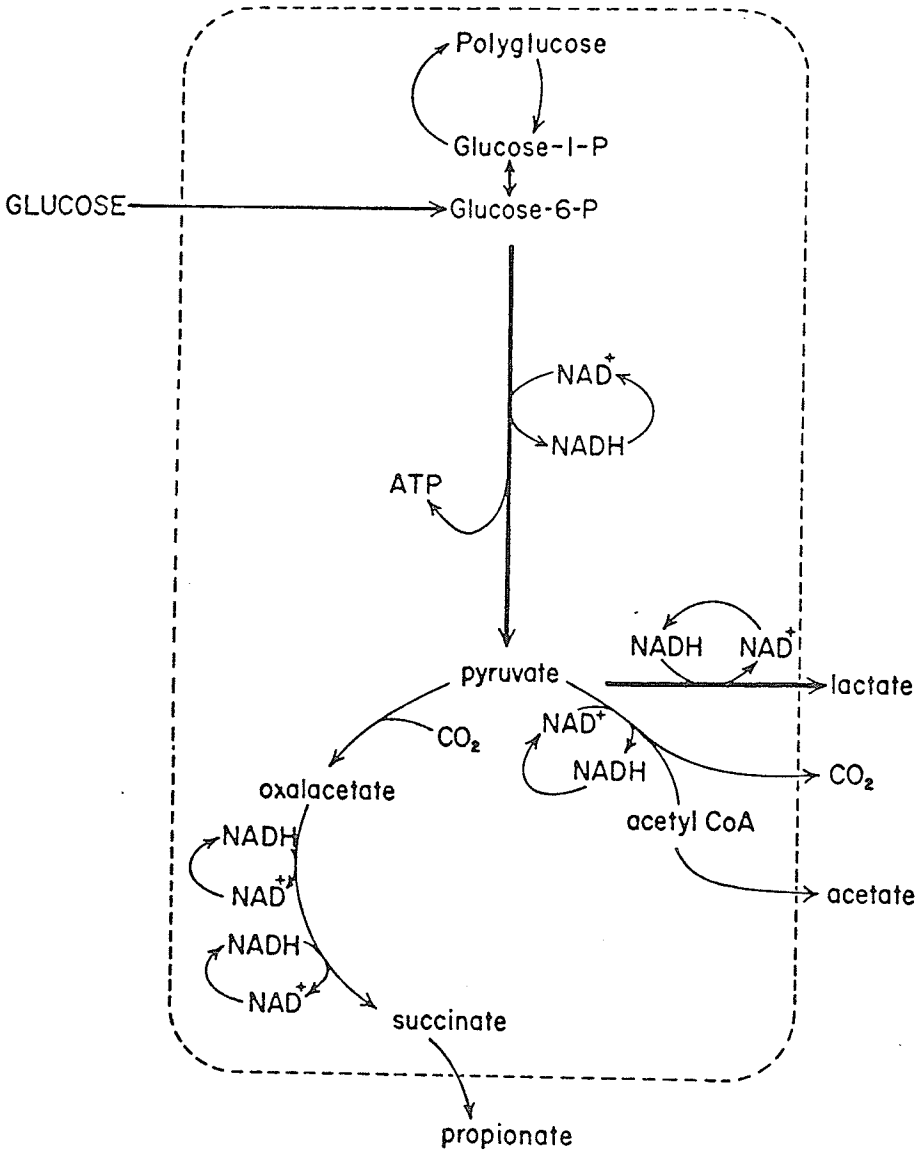


Fig. 1.2. Pathways proposed for the metabolism of glucose by salivary sediment. (From, Sandham and Kleinberg, 1970b)

STEPHEN (1943), found that all plaque samples from a large number of individuals showed ureolytic activity. However, the microorganisms within the plaque responsible for its ureolytic activity have not been established, (FROSTELL, 1960).

The most active organisms in terms of ureolytic activity seem to be the micrococci (FROSTELL, 1960). However, the ureolytic properties of suspensions of such oral isolates are different from those of plaque (FROSTELL, 1959, 1960, 1962). Whereas the pH optimum for ammonia production from urea by the micrococci was on the acid side of neutrality, plaque suspensions showed a broad optimum between pH 7.0 and 8.0. Also, the effect of urea concentration on ammonia formation by suspensions of plaque and micrococci was different. Ammonia production by plaque tended to reach optimum levels when the urea concentration was between 0.2 M and 0.4 M whereas with pure cultures, ammonia formation was much less than maximum at such urea concentrations. In this regard, the mixed populations of the salivary sediment system (BISWAS and KLEINBERG, 1971) were also different than the micrococci and behaved similar to plaque since near maximum ammonia formation occurred at a urea concentration of 0.28 M.

Not only does the ureolytic activity of the mixed oral flora differ from that of pure cultures of ureolytic microorganisms, but the numbers of ureolytic microorganisms isolated from plaque is much too small to account for the level of ammonia production by plaque suspensions (FROSTELL, 1960). This suggests that either the cultural conditions employed to isolate the ureolytic bacteria from plaque are inadequate, or bacterial interactions within the mixed population may enhance overall ureolytic activity, or ureolytic mechanisms in the plaque may not be the same as in the pure cultures.

In support of the latter possibility are the observations of BISWAS and KLEINBERG (1971) with the mixed oral flora of the salivary sediment system. These investigators found that in the presence of urea the formation of NH_3 and CO_2 does not occur in the 2:1 ratio which might be expected if urea was hydrolyzed by the enzyme urease. Rather, the degradation of one mole of urea resulted in the formation of nearly one mole of CO_2 but only about 0.2 moles of NH_3. Apparently, NH_3 was readily assimilated and stored while CO_2, though transiently stored, was almost completely released. ^{14}C-urea tracer studies showed that small amounts of urea-C were incorporated into lactate and succinate. When the sediment cells were disrupted by ultrasonication, the sonicates failed to utilize urea. This was interpreted to indicate that a more complex sequence for urea degradation exists, rather than the action of a "single" enzyme such as urease. Therefore, it was proposed that urea is degraded by a reversal of the ornithine cycle in such a manner that the majority of urea-N would be incorporated into amino acids while most urea-C would be released as CO_2, Fig. 1.3 (BISWAS and KLEINBERG, 1971).

(ii) in vivo studies

The immediate and rapid rise in the pH of dental plaque in situ (KLEINBERG, 1967) following a urea rinse indicates that an active ureolytic mechanism exists in dental plaque. The effect of urea concentration on the pH change is similar to that one would expect of an enzyme system (KLEINBERG, 1967), since an asymptotic pH response is observed with increase in the concentration of urea.

Salivary urea appears to be an important determinant of the steady state pH of fasting plaque in situ (KLEINBERG and JENKINS, 1964).

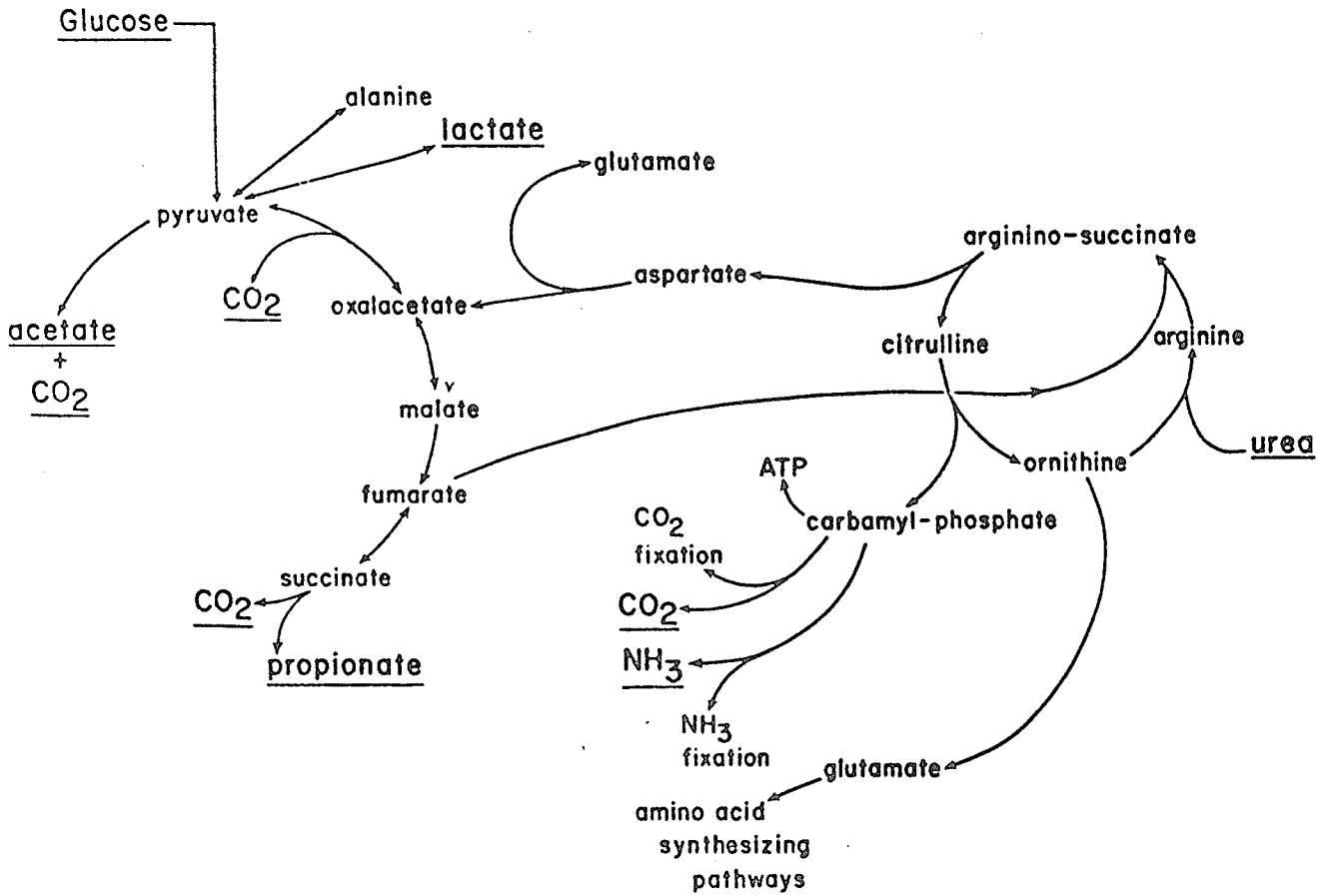


Fig. 1.3. Pathways proposed for the metabolism of urea and glucose by salivary sediment. (From, Biswas and Kleinberg, 1971).

The plaque pH was higher than that of the saliva bathing it. This is attributed to the rapid conversion of the salivary urea to base and the slow loss of the base from the plaque into the saliva. Furthermore, plaques with greater salivary exposure had higher pH levels; this was attributed to the greater base formation that would occur with increased availability of salivary urea. In support of the hypothesis that salivary urea is an important determinant of plaque pH was the finding that the levels of urea normally present in saliva when applied to plaque in situ (but not amino acids or protein; viz.FROSTELL, 1960) caused a pH response of sufficient magnitude to account for the pH difference between plaque and its bathing saliva (KLEINBERG, 1967).

(c) Proteins and peptides

Proteolytic activity has been demonstrated in whole saliva (MAKINEN, 1966 a), parotid and submaxillary saliva (SODER, 1972), subgingival plaque (LUCAS and THONARD, 1955) and supragingival plaque (MAKINEN, 1966 b; SODER and FROSTELL, 1966; SODER, 1972). Some question seems to exist whether most of this activity is of bacterial origin or derived from secretions of the salivary glands (SODER, 1972). While parotid saliva had relatively weak proteolytic activity, such activity was high in submandibular saliva and extracts of supragingival plaque. Furthermore, when both submandibular saliva and extracts of plaque were subjected to the technique of iso-electric focussing, the fractions showing proteolytic activity tended to coincide.

Extracts of plaque contain both extracellular endopeptidases (SODER, 1967) which attack peptide bonds within the polypeptide chain, and exopeptidases (MAKINEN, 1966 b) which attack amino acids in the end positions. Gel filtration chromatography of plaque extracts has revealed as many as three different fractions with proteolytic activity (SODER,

1966). KLEINBERG (1970 a) has shown that when salivary sediment is incubated at low pH, proteins having a low isoelectric point disappear from the medium. On the other hand, when incubated at high pH, the more basic proteins are degraded. This indicates that at least two types of proteases are active in salivary sediment, (viz. SODER, 1966). One type acting on acidic proteins with its optimum activity at acidic pH and another acting upon the more basic proteins with its optimum activity at alkaline pH. These types of proteolytic reactions may supply the peptides and amino acids which are considered important in the metabolism of the mixed oral flora (MOLAN and HARTLES, 1971).

A substance present in whole saliva, referred to as "pH-rise factor" (CRAW et al., 1968) and tentatively identified as a peptide, influences the metabolism of the mixed oral flora (KLEINBERG et al., 1968; KLEINBERG, 1970 a). This substance, which may be the same as the glycolysis enhancing factor observed in saliva by HARTLES and WASDELL (1955), stimulates the uptake of glucose and facilitates the subsequent formation of base by the salivary sediment (KLEINBERG, 1970 c).

(d) Amino acids

Amino acids are found free in saliva (KIRCH et al., 1947; WALDRING, 1955; BATTISTONE and BURNETT, 1961), gingival crevice fluid (BRILL, 1962) and dental plaque (BLACKWELL et al., 1954). In addition to duct saliva (ROSE and KREE, 1958; BATTISTONE and BURNETT, 1961), the amino acids in whole saliva may also arise from the breakdown of protein in the saliva or in plaque (FROSTELL and SODER, 1970; CRITCHLEY, 1969).

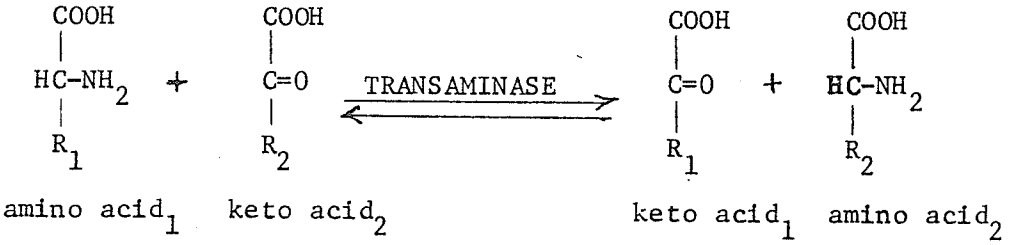
Urea can act as a nitrogen source for the formation of amino acids by the bacteria in salivary sediment (BISWAS and KLEINBERG, 1971). In the presence of glucose and urea considerable formation of alanine occurs (BISWAS and KLEINBERG, 1967), the urea, presumably supplying the amino

group for transamination of pyruvate derived from the glucose.

Depending upon the pH, amino acids may be further metabolized by either one of two enzymic reactions (GALE, 1946). At high pH, deamination of amino acids can occur and result in the formation of ammonia and keto-acids. On the other hand, at acidic pH, amino acid decarboxylation is favoured, resulting in the formation of carbon dioxide and amines. Amino acid decarboxylase activity has been demonstrated in salivary sediment (GOCHMAN et al., 1959). This activity was most pronounced with ornithine and arginine, though some activity was evident with glutamic acid, histidine and lysine.

It has been suggested that deamination and decarboxylation are important homeostatic mechanisms in the acid-base metabolism of the oral flora (KLEINBERG, 1970 c). Since keto-acids formed during deamination would be stronger acids than ammonia is a base, the pH would decrease. On the other hand, the amines formed during decarboxylation reactions would cause the pH to rise.

Transaminases reactions play an important role in the synthesis of most amino acids (LAMANNA and MALLETTE, 1965). These enzymes function in the transfer of amino groups from one amino acid to another. The general process of transamination may be represented as:



While there have been few studies of these types of reactions by the mixed oral flora, transaminase activity has been reported by saliva (MARTINELLI and PELLEGRINI, 1957; DRIEZEN et al., 1969; KRONCKE, 1961).

In comparisons of the glutamic-oxaloacetic (GOT) and glutamicpyruvic (GPT) transminase concentrations in paraffin-stimulated whole saliva and serum of healthy subjects, DRIEZEN et al., (1959) found that the GOT content of saliva exceeds that of serum while serum GPT is higher than that of saliva. Because there was no significant correlation between the serum and salivary values, these investigators suggested that the oral flora and oral tissues may contribute to the transaminase content of saliva. When whole saliva was incubated with glucose there was a marked elevation of the GPT activity indicating that this enzyme may be important in the synthesis of amino acids by salivary micro-organisms during glucose catabolism.

Factors Important in Comparing the Metabolisms of in vitro Bacterial Systems to Dental Plaque in situ

(a) Cell concentration

Most studies concerning the metabolism of oral bacteria have used saliva or pure cultures in which the cell concentration was low. Since the cell concentration in plaque is very high, STEPHAN and HEMMENS (1947) investigated the effects of cell concentration on the pH changes produced by pure cultures of different oral microorganisms suspended in a buffer resembling saliva and containing glucose (0.01M) as a substrate. The cell concentrations were varied from 1 to 33 percent of the total volume. In general, it was found that only at the higher cell concentrations did the pH curves resemble those which occur with plaques in situ. It was also found that the plaque isolates varied not only in their ability to produce acid but also in their ability to "consume" it. At higher cell concentrations both the pH drop and subsequent pH rise occurred more quickly.

The lowest pH attained during incubation was not achieved at the same cell concentration for each of the organisms examined. Some achieved their lowest pH levels at high cell concentrations, some at intermediate cell concentrations, while others reached this point at low cell concentrations. STEPHAN and HEMMENS suggested that these differences may be due to a variation in the relative rates of acid production and acid consumption with different cell concentrations.

STRALFORS (1950), who also examined the effect of cell concentration, demonstrated that the slow fall in pH with saliva-glucose mixtures as compared to the rapid pH decrease in dental plaque could be accounted for by the low initial concentration of micro-organisms in saliva as compared to plaque. In a culture medium containing glucose he found that as the bacteria increased in numbers the total amount of acid produced also increased. However, as acid accumulated and the pH fell, the actual rate of acid production decreased. These effects were different for the different bacteria examined. For example, acid production by lactobacilli occurred at a slower rate than that for streptococci when examined at high pH (pH range 6.5 - 7.0); the converse was found at low pH values (pH range 4.5 - 5.0). This effect of pH on the ability of different bacteria to utilize glucose and produce acid may also have some bearing on the differences in pH minima achieved by different concentrations of the organisms studied by STEPHAN and HEMMENS (see above).

KLEINBERG (1967) also studied the effect of cell concentration; however, in this case a mixed cell population was used. The cells were the mixed oral flora which constitute the sediment of paraffin stimulated whole saliva. In this study pH-glucose concentration curves at different sediment concentrations (0 - 33%) were examined and compared to the pH-

glucose concentration curves determined for plaque *in situ* (KLEINBERG, 1961). Only at the higher sediment concentrations were the pH-glucose concentration curves similar to those of plaque *in situ*. Similar to results with plaque *in vivo*, the pH-time curves with the high concentrations of sediment exhibited a fall and rise, the extent of the former and the time of occurrence of the latter being determined by glucose availability. The pH curves for plaque *in situ* and sediment *in vitro* were similar in character when sediment, substrate concentrations and system conditions were adjusted. These concepts will be discussed in greater detail below.

(b) Substrate availability

When glucose is added to the suspended salivary sediment (SSS) system, different types of pH curves are obtained depending upon the concentration of added substrate (KLEINBERG, 1967). With low glucose concentrations, the glucose is used up before the end of the four hour incubation period. As a result, the pH falls rapidly, reaches a minimum and then slowly rises.

With glucose concentrations sufficiently high to maintain glucose for long period of time, the pH falls initially rapidly then instead of rising, it continues to fall at a progressively slower and slower rate. It is apparent that in the closed *in vitro* SSS system that substrate availability is determined by the initial substrate concentration. In such a closed system the removal of acidic or basic end-products and the return of the pH to base-line levels can only occur by biochemical processes within the system (KLEINBERG, 1970). Plaque *in situ*, on the other hand, is usually an open system. As described by KLEINBERG, (1961), the characteristics of such a system require that substrate availability be determined mainly by the length of time that a given substrate

concentration is available to the plaque microflora and to a lesser extent by the initial substrate concentration. Furthermore, the removal of acidic or basic end-products from plaque *in situ* may be aided by saliva as well as the biochemical processes. Therefore, in order to obtain pH curves in the SSS system *in vitro* with characteristics similar to those observed in plaque *in situ*, both the cell concentration and the availability of substrate must be adjusted.

Presence and Source of Urea, Ammonia and Amino Acids in Saliva and Dental Plaque

(a) Urea

The presence of urea in saliva (Table 1.2) is not too surprising since urea is a highly diffusible substance and is a normal blood constituent. In fact, the urea concentration of the parotid gland and nearly all other organs and tissues of the body is quite close to that of blood (MARSHALL and DAVIES, 1914).

One of the earliest reports of urea levels in saliva (HENCH and ALDRICH, 1922) indicated that due to bacterial degradation the combined urea-N and ammonia-N values are probably a more accurate reflection of the urea concentration in mixed saliva than urea-N values. In normal adults, the urea concentration of mixed saliva was approximately 80 percent of that found in blood (HENCH and ALDRICH, 1923). When urea was fed by stomach tube, both the salivary and blood urea levels increased and then decreased in a parallel fashion and maintained their usual concentration difference (HENCH and ALDRICH, 1923). The parallel behaviour of saliva and blood urea levels has also been demonstrated in urea retention disease states (SCHMITZ, 1922) and in children (NIKIFORUK, 1956).

The concentration of urea in mixed whole saliva (BARNET and

TABLE I.2. Urea content of saliva

Type	urea mg%		reference
unstimulated	average	range	
whole saliva	20	13 - 27	Hench and Aldrich, 1922
	44.9	14.4 - 75.0	Barnet and Bramkamp, 1929
	20	____	Stephan, 1943
parotid saliva	29.3	11.9 - 45.4	Barnet and Bramkamp, 1929
	28.0	____	Shannon and Prigmore, 1960
stimulated			
whole saliva	22.7	11.6 - 29.8	Barnet and Bramkamp, 1929
	13.0	____	Stephan, 1943
parotid saliva	22.1	9.1 - 27.6	Barnet and Bramkamp, 1929
	18.8	____	Shannon and Prigmore, 1960

BRAMKAMP, 1929) and in parotid saliva (BARNET and BRAMKAMP, 1929; ALBRECTSEN and THAYSEN, 1955; SHANNON and PRIGMORE, 1960) is inversely related to the flow rate. Prolonged salivation does not significantly affect salivary urea levels (HENCH and ALDRICH, 1922; SHANNON and PRIGMORE, 1960).

More recently, a potentially important source of urea to dental plaque has been identified as that present in the gingival crevice fluid (GOLUB et al., 1971). Urea concentrations as high as 300 mg% have been demonstrated in fluid from "clinically" healthy crevices. As inflammation and pocket depth increases the urea concentration decreases approaching that of the blood (19 - 33 mg%; DALTA, 1968).

Reports regarding the presence of urea in dental plaque are not available.

(b) Ammonia

The concentration of ammonia in duct saliva is apparently very low (SCHMITZ, 1922; BRAMKAMP, 1937) and is much less than its concentration in whole saliva (SCHMITZ, 1922; CARY, 1946). HENCH and ALDRICH (1922) considered the ammonia in whole saliva to arise from urea breakdown by the bacteria in the oral cavity. The fact that ammonia-N was greater in saliva collected from an unwashed mouth than from a washed mouth and the finding that the combined urea and ammonia-N values in both cases were nearly constant were the bases for this conclusion. SCHMITZ (1922) found that incubated, filtered (Berkfield filter) whole saliva did not exhibit a decrease in urea or an increase in ammonia as did unfiltered saliva. He concluded that salivary ammonia was derived from "bacterial action on urea and not by an enzyme" (i.e. not a salivary enzyme).

The ammonia content of unstimulated whole saliva of healthy

subjects has been reported to be in the range 0.0 to 26 mg percent (YOUNGBERG, 1936).

Besides urea, the degradation of amino acids and amino sugars also may produce ammonia (FROSTELL, 1960). The degradation of glucosamine by saliva has been demonstrated by ROGERS (1948), while KESEL et al., (1947) found deaminase activity in saliva.

LUDWICK and FOSDICK (1950) appear to have published the only report showing that ammonia is present in dental plaque. However, their methods did not allow for its quantitation since the amount of plaque collected was not determined; another difficulty is that their samples were contaminated with saliva.

(c) Amino acids

KIRCH et al., (1947), using microbiological assay techniques, identified sixteen amino acids in paraffin stimulated whole saliva. In subsequent studies, these investigators unsuccessfully attempted to relate the protein content of the diet with the salivary free amino acids content (KIRCH et al., 1950). However, they did find for several amino acids that the ingestion of a single amino acid significantly increased that particular amino acid in whole saliva for a period of 30 to 60 minutes (KIRCH et al., 1953).

With the advent of chromatographic techniques GOLDBERG et al., (1948), FOSDICK and PIEZ (1953), WALDRING (1955), ROSE and KERR (1958), BATTISTONE & BURNETT (1961) confirmed that most, if not all, of the common amino acids can be detected in whole saliva. Although levels of the various amino acids are quite variable, the predominant amino acids appear to be glycine, glutamic acid, alanine and lysine.

The concentration of amino acids in parotid saliva is quite low (BATTISTONE and BARNETT, 1961). In contrast, the amino acid content

of stimulated submandibular saliva is higher; it is qualitatively quite similar to that of stimulated whole saliva and may contain from 30 to 50 percent of the total amino acids of whole saliva (BATTISTONE and BURNETT, 1961).

The free amino acids of dental plaque have been studied to a much lesser extent. BLACKWELL et al., (1954) using ethanol (75% V/V) to extract the plaque free amino acids, found arginine, alanine, aspartic acid, glutamic acid, glycine and valine to be the amino acids most frequently present. Leucine, isoleucine and proline were usually present and on occasion lysine, taurine, tyrosine could be detected. Histidine, tryptophan, threonine and phenylalanine could only be detected in pooled plaques from more than one individual, indicating their relatively low concentrations.

CRITCHLEY (1969), using cold deionized water as the extractant, attempted to extract the free amino acids from the plaque matrix. He stated that "very little material was extracted from the cells in plaque" since acid hydrolysis and paper chromatography failed to demonstrate the presence of rhamnose (a sugar typical of streptococcal cell walls) or ribose (from ribonucleic acid). This reasoning is erroneous since the free amino acid pools of gram negative bacteria are very labile and may be extracted quite easily with cold deionized water (HOLDEN, 1962), whereas, rhamnose and ribose are parts of cell macromolecules that are not likely to be easily extracted.

Ureolytic microorganisms in the oral cavity

In the human mouth the ability to degrade urea is apparently confined to the bacteria (SCHMITZ, 1922; CARY, 1946). HINE and O'DONNELL (1943) found microorganisms with ureolytic activity in 92

percent of samples of unstimulated saliva taken from 82 patients. In every sample showing marked activity, bacteria of the proteus group, and streptococci and staphylococci (micrococci) with ureolytic capability were isolated.

Streptococci with such capability have also been isolated from human mouths by ONISI (1957) and SMITH and BODILY (1968). The last of these investigators found that, of the salivary microorganisms showing ureolytic activity, 14 percent were streptococci and 81 percent were micrococci. Further, of 109 strains of aciduric micrococci isolated from paraffin stimulated whole saliva (JORDAN et al., 1956), 76 were able to degrade urea.

The main ureolytic organisms of dental plaque also appeared to be the micrococci (FROSTELL, 1960). Under aerobic conditions, MASUDA (1958 a) isolated 176 strains of bacteria from dental plaque. Of these, 30 percent exhibited ureolytic activity. The numerical distribution of the microorganisms responsible for this activity was: Sarcina flava (40 percent); Micrococcus conglomeratus (18 percent); Micrococcus varians (11 percent); Micrococcus pyogenes var. aureus (9 percent); Neisseria flava (7 percent); Micrococcus pyogenes var. albus (7 percent); Bacillus subtilis (7 percent); Vibris tyrogenu (2 percent); Aerobacter cloacae (2 percent) and Corynebacterium pseudodiphtheriticum (2 percent). The rate of ammonia production from urea was highest by far for Micrococcus pyogenes var. aureus. This strain exhibited ammonia production activity ten times that of Sarcina flava and 100 to 1000 times that of the other ureolytic organisms isolated.

The high ureolytic activity of micrococci has been confirmed by FROSTELL (1959 and 1960). FROSTELL, however, was unable to isolate a Proteus strain from the oral cavity nor demonstrate that aerobically

grown streptococci, diphtheroids or neisseriae have significant ureolytic activity.

Several factors may be responsible for the difference in results between the various investigators. Firstly, most of the studies used complex culture media containing a source of readily utilizable carbohydrate. However, carbohydrate in the culture medium can either stimulate or inhibit ureolytic activity (MASUDA, 1958 b). In this regard carbohydrate was indispensable for the production of ureolytic activity in Micrococcus pyogenes var. aureus but for Sarcina flava carbohydrate inhibited the activity. Secondly, as pointed out by FROSTELL (1960) ureolytic activity cannot be certain unless no ammonia is produced when urea is absent. This is particularly pertinent in evaluating the studies of HINE and O'DONNELL (1943) and SMITH and BODILY (1968) since whole saliva was in the culture media and although the culture contained urea, it also contained salivary proteins which could also be a source of ammonia (KLEINBERG, 1970 a).

Role of Urea and Ammonia in Caries, Calculus Formation, and Periodontal Disease

(a) Caries

Ammonia formation from urea has been extensively investigated in relation to dental caries. For example, GROVE and GROVE (1934, 1935) found markedly higher levels of ammonia in the salivas of caries free than of caries active subjects, whereas other investigators could find no relationship (WHITE and BUNTING, 1935; YOUNGBERG, 1936; KARSHAN, 1936). KESEL et al., (1949) found that saliva from individuals who were caries-resistant had more urea and formed ammonia from urea more rapidly than did persons who were caries active. However, JENKINS and WRIGHT (1950)

could find no difference in ammonia formation.

KESEL et al., (1949) identified salivary ammonia as a growth inhibitory factor of lactobacilli and other acid-producing organisms in fresh saliva (KESEL et al., 1946, 1947, 1949). This effect of ammonium ions on growth of oral lactobacilli could not be confirmed by KIRCHHEIMER and DOUGLAS (1950) nor by JENKINS and WRIGHT (1951). The latter investigators showed that high concentrations of ammonium salts were only slightly more active than other salts in inhibiting lactic acid formation from glucose in the mixed bacteria of saliva or in pure cultures of lactobacilli. On the other hand, with low concentrations of ammonium salts stimulation of acid production occurred.

In animal studies, 5 percent urea in a cariogenic diet fed to rats (MASUDA et al., 1968; FROSTELL, 1970) or hamsters (FROSTELL, 1970) resulted in a significant reduction in the incidence of caries as compared to control animals. Surprisingly, 50 percent urea painted on the teeth of hamsters at weekly intervals actually increased the incidence of caries considerably (KEYES, 1946).

Urea applied to plaque in situ can prevent the decrease in pH that occurs following glucose (STEPHAN, 1943) or sucrose exposure (FROSTELL, 1969). STEPHAN and MILLER (1944) in a clinical investigation of the anti-caries effect of an urea rinse found an 80 to 100 percent reduction in caries incidence with a 45 percent urea solution.

A reduction in caries incidence has also been demonstrated in clinical studies with dentifrices containing urea and ammonia (KESEL et al., 1947; HENSCHEL and LIEBER, 1952; LEFKOWITZ and VENTI, 1951). However, because of certain deficiencies in the experimental design of these trials and based upon their own studies, HAWES and BIBBY (1953)

concluded that such dentifrices are not active as caries inhibiting agents.

A comprehensive test of the effects of urea and/or ammonia on caries reduction is still needed (REGOLATTI, 1971) if the role of urea in caries inhibition is to be resolved.

(b) Calculus

Ammonia formation has been examined often in relation to calculus formation. FRANK (1929) found a positive correlation between the incidence of calculus and the salivary ammonia concentration and concluded that high ammonia levels are the cause and not the effect of calculus formation.

The production of ammonia from patients with and patients without calculus has also been measured (JACOBSON, 1950 a, b; JACOBSON and KESEL, 1950). During a 72 hour incubation period, ammonia production was greater in saliva from patients with calculus than from those who were caries-free. While such prolonged incubation periods are conducive to the formation of ammonia from sources other than urea (e.g. salivary proteins; KLEINBERG, 1970 a), GOMEZ (1968) found that the ureolytic activity of whole saliva also is greater in individuals with abundant calculus. Furthermore, the urea concentration in submandibular duct saliva is significantly increased in calculus-formers as compared to non-formers (MANDEL and THOMPSON, 1967). These latter authors suggested that ammonia per se is not necessarily important in calculus formation. Rather, under conditions where a receptive matrix and sufficient levels of calcium and phosphate are present, a local elevation of pH (associated with ammonia formation from urea) would favor the mineralization of plaque.

The effect of urea as a possible inhibitor of calculus formation

has been investigated by BELTING and GORDON (1966 a and b). These investigators tested the effect of increasing concentrations of urea on the removal of calcium containing deposits formed from saliva on glass slides. Deposition was inhibited (70 percent) and removal was maximum at a urea concentration of 30 percent. In vivo experiments in which subjects used a toothpaste containing 30 percent urea over a fourteen day period also resulted in reduced calculus formation. These investigators concluded that urea at high concentration may favour the solubilization of the organic matrix or the inorganic components of calculus.

However, McGAUGHEY and STOWELL (1967) have shown that 36 percent urea solutions do not affect either desorption or adsorption of salivary proteins by hydroxyapatite. MUKHERJEE (1969) has found that in ten minutes, 30 percent urea can dissolve about 3.4 percent of the total phosphorus in fully formed calculus. This is not likely to be significant unless the dissolution was carried out over an extended period or while calculus is forming. The experiments of KLEINBERG et al., (1971) suggest another way for high urea concentration to inhibit calculus formation. These investigators had subjects rinse with a 10 percent urea solution for one minute three times a day during the fromation of three-day plaque and found that plaque formation itself was inhibited.

(c) Periodontal disease

The pH pattern of fasting plaques in situ has been related to the intra-oral incidence of periodontal disease and may be due to variation in salivary urea availability (KLEINBERG and JENKINS, 1964). A similar pattern for the incidence of ureolytic organisms in plaque sampled from various interdental areas throughout the mouth has been

described by ONISI et al., (1957).

Plaques from patients suffering from periodontal disease show higher ureolytic activity than plaques from healthy persons (FROSTELL, 1960). Differences in ammonia production capacity, i.e., the product of the amount of plaque material collected from a patient's teeth and its ammonia production activity amongst such patients are even greater (FROSTELL, 1960). Ammonia production capacity was nearly three times larger in patients having periodontitis than in those with a healthy periodontium.

Urea is also present in gingival crevice fluid (GOLUB et al., 1971) and by its conversion to NH_3 by bacteria could account for the high pH within such crevices (KLEINBERG and HALL, 1969). Whether or not the high urea concentration of fluid from minimally inflamed crevices is beneficial or not in terms of gingival health could not be decided (GOLUB et al., 1971).

Plaque Formation

The first structure which appears on the surfaces of the teeth after prophylaxis is a bacterial-free cuticle about 1,000 A^{o} thick (FRANK and HOUVER, 1969). This acquired pellicle (DAWES et al., 1963) is apparently of salivary origin (MECKEL, 1965; MANDEL, 1966; THEILADE and MIKKELSEN, 1972) and may be important for initiation of plaque formation (MECKEL, 1971).

Using the scanning electron microscope, SAXTON (1973) noted that within the first few hours after a prophylaxis discrete globules of bacteria formed upon enamel surfaces adjacent to those margins of gingivae showing some degree of inflammation. Bacteria were not

evident in such deposits for at least three hours after the prophylaxis, if the gingivae were healthy. After 24 hours of plaque development, the organisms residing within the enamel defects were able to proliferate and invade the dental plaque. Thereafter, extension of the globules or "colonies" resulted in coverage of large portions of the tooth surface. After several days, this plaque was devoid of specific topographical characteristics when viewed under the stereomicroscope (BJORN and CARLSSON, 1964).

(a) Role of salivary protein

KIRK (1910), DOBBS (1932) and others, have suggested that the initiation of plaque formation occurs when acid produced by the oral bacteria causes the precipitation of mucin from saliva. WINKLER and BACKERDIRKS (1958) proposed that salivary mucoid after contacting the oral surfaces becomes denatured and forms deposits to which bacteria adhere. The production of acid by the bacteria adhering to this layer could then precipitate further mucoid and thus facilitate the build-up of the dental plaque.

Spectrophotometric-titration studies (TRESTER and KLEINBERG, 1962; KLEINBERG et al., 1971) have shown that although the rate of precipitation of salivary protein occurs most readily at acidic pH, precipitation occurs over a much wider pH range (pH 4.0 to 10.0).

Though proteins may deposit on objects dipped in sterile duct saliva (MIDDLETON, 1965), LEACH (1970) has concluded on the basis of several enzymic and compositional studies (LEACH, 1964, 1965; LEACH and CRITCHLEY, 1966; LEACH and MELVILLE, 1970) that plaque may first form as a result of the action of bacterial extracellular enzymes on salivary glycoproteins causing these proteins to precipitate. While salivary mucin has a low iso-electric point, loss of sialic acid from

this protein by the action of neuraminidase raises the pH of minimum solubility (iso-electric point) towards pH 7.0 and could result in a tendency for these macromolecules to precipitate. The lack of detectable sialic acid in dental plaque (DAWES and JENKINS, 1963) and the ability of certain oral bacteria to produce neuraminidases (LEACH and HAYES, 1967; LEACH and MELVILLE, 1970) tend to support this view. However, recently BRISCOE et al., (1972) have found that while aggregates form in saliva treated with neuraminidase, such treatment does not increase the affinity of salivary proteins for hydroxyapatite powder with or without previously adsorbed salivary components or neuraminidase.

(b) Specific adherence of oral bacteria

HILLMAN et al., (1970) found that various species and strains of oral bacteria differ in their ability to adhere to either untreated or saliva-coated enamel powder. Whereas some bacteria adhered well to both (Leptotrichia buccalis strain Le), other preferentially adsorbed to one (Actinomyces naeslundii strain I) or the other (Strep. sanguis strain od 1). Of the bacteria examined only a Lactobacillus casei strain failed to adhere to either preparation.

Studies in vivo (Van HOUTE et al., 1970 and 1971) have indicated that Strep. sanguis has a greater affinity for clean tooth surfaces or for dental plaque than Strep. salivarius, but a lesser affinity for the dorsum of the tongue. On the basis of such studies, LILJEMARK and GIBBONS (1971, 1972) have proposed that the relative affinity of oral bacteria for different oral surfaces will determine their occurrence at such sites.

(c) Polymer-bacterial interactions

SILVERMAN and KLEINBERG (1967) separated plaque into cellular and acellular components using cold 0.1N NaOH. Using these fractions in re-aggregation studies, they showed that the factors favouring re-aggregation of the cells were (i) an acidic pH, (ii) the presence of calcium, (iii) an increase in the ionic strength, and (iv) the presence of a macromolecular carbohydrate-protein fraction of the acellular component of plaque which had a high calcium and phosphate content.

Indirect evidence suggests this latter material is deposited from saliva during plaque formation even when the pH is weakly acidic (KLEINBERG et al., 1971). Furthermore, x-ray diffraction analysis has shown that the precipitate of such salivary aggregates has an intense reflection at 4.15 A^{o}, such a reflection is also present in the diffraction pattern of early plaque (KAUFMAN and KLEINBERG, 1973). On the basis of these findings KLEINBERG et al., (1971) suggested that during plaque formation a floc-like aggregate of salivary calcium phosphate carbohydrate-protein and bacteria would form on the tooth surface. Growth of the bacteria and formation of acids from dietary carbohydrate would hasten this process and result in continued deposition of salivary-protein-bacterial aggregates. In agreement with this concept, GIBBONS and SPINELL (1969) found that the aggregation of many bacteria isolated from dental plaque was encouraged by saliva and that calcium was required for this action. KASHKET and DONALDSON (1972) suggest that many different aggregation factors exist in saliva, each of which may be capable of interacting with cells of one of several bacterial species. However, failure to control many of the experimental variables in their system makes it difficult to decide on the validity of these conclusions.

GIBBONS and FITZGERALD (1969) have proposed that extracellular dextrans of bacterial origin may be necessary for the formation of plaques by Strep. mutans, since in culture these organisms form large amounts of these polymers from sucrose and form "plaque" in animals provided with high levels of dietary sucrose (FITZGERALD and JORDAN, 1968). Since glucose grown cells can be aggregated by the addition of these polymers, GIBBONS and FITZGERALD (1969) postulated that dextrans present on the tooth surface facilitate the selective adherence of Strep. mutans by interacting with dextransucrase, an enzyme bound to the surface of these organisms. According to these authors, the "gelatinous"* plaques characteristic of high sucrose intake may be initiated in this manner.

However, KLEINBERG et al., (1971) have pointed out that such a mechanism would require that dextran formation occur simultaneously with the maintenance of the dextran producing sites free of dextran. As this is not probable, these authors suggested that a simpler hypothesis would be that salivary bacteria with attached dextran become attached directly to the tooth surface and in the presence of dietary sucrose multiply and form a "gelatinous" type of plaque.

*Note: gelatinous is a term used in the literature for gross morphological description of such plaques. No collagen or denatured collagen has been identified in plaque.

Nature of the Bacterial Surface

(a) Chemical Composition

The main structural component of the bacterial periphery is the bacterial cell wall which is a rigid water-insoluble envelope surrounding the cytoplasmic membrane. It consists of a peptidoglycan complex, also called mucopeptide or murein. The peptidoglycan is

composed of two different N-acetylhexosamines, N-acetyl-D-glucosamine and N-acetylmuramic acid, and a few amino acids. The amino acids are usually alanine, glutamic acid and a dibasic amino acid, most often lysine or α-diaminopimelic acid (OSBORN, 1969).

Bacterial cell walls, however, are never pure peptidoglycan (GHUYSEN et al., 1968). In most gram-negative bacteria, the non-peptidoglycan components represent as much as 90 percent of the weight of the cell walls and may be protein (in Streptococcus) polymers of polyphosphate (techoic acids in various strains of Streptococcus, Staphylococcus, Bacillus and Lactobacillus), polymers of N-acetyl-phostosamine and glucuronic acid (teichuronic acid on Bacillus subtilis), or polymers of L-rhamnose and N-acetyl-D-glucosamine (polysaccharide C in Streptococcus pyogenes).

McCARTY (1971) describes the cell wall of Group A Streptococci as a three-layered structure: the inner layer being the peptidoglycan, the middle layer consisting of the group-specific polysaccharide (rhamnose and N-acetylglucosamine), while the outer layer is thought to be the protein antigens (type-specific M, T, and R proteins).

In certain streptococcal groups (especially groups A and C), capsules of hyaluronic acid are detectable in the early exponential phase. However, as the organisms continue to grow logarithmically, hyaluronidase is produced and the capsules are no longer detectable (KASS and SEASTONE, 1944).

In the presence of sucrose, certain strains of oral streptococci form heavy polysaccharide capsules (NOLTE, 1973).

(b) Surface Properties

While chemical studies have yielded information regarding

the components of bacterial cell walls and capsules, it is less clear how these components are structurally arranged and thus how they may participate in such surface dependent phenomena as aggregation and adhesion.

(i) Microelectrophoretic studies

Many studies examining the behavior of bacterial surfaces have used the technique of microelectrophoresis. A finding common amongst these studies is that bacterial cells have a net negative charge at neutral pH. The origin of this charge has been attributed to ionization of surface ionogenic groups (JAMES, 1965) which are associated with the bacterial cell wall (NEIHOF and ECHOLS, 1968; HILL et al., 1963; JAMES and BREWER, 1968).

Since the surface of bacteria can have a complex composition (see above) the substances that give rise to surface charges may be variable. The charge on protein molecules is mainly determined by their ionizable groups, since adsorption of ions is limited by hydration. On the other hand, the charge on lipid molecules is mainly due to adsorption of ions since they are unhydrated and possess fewer ionizable moieties. Since polysaccharide molecules are hydrated and have few ionizable groups their charge is small. The surface charge characteristics of bacterial cells could correspond with either of these substances or combinations of them. Thus, some bacteria behave as if their surfaces are composed of non-ionogenic areas interspersed with a small number of ionogenic groups (HAYDEN, 1961), others as if ionogenic groups are the main determinants (ABRAMSON, 1934; GITTENS and JAMES, 1963; JAMES, 1965) and still others as if ionogenic groups are absent (ABRAMSON, 1934).

The iso-electric point for most bacteria lies between about pH 1.0 and 4.6 (DAVIES, HAYDEN and RUDEAL, 1956; ABRAMSON, 1934). Ionogenic groups which can contribute to the surface charge are the carboxyl, phosphate and amino groups (PLUMMER and JAMES, 1961; HILL et al., 1963; JAMES, 1965; NEIHOF and ECHOLS, 1968; ECHOLS and NEIHOF, 1969). The negative charge of the bacterial surface can be reduced not only by lowering the pH of the suspending medium, but also by the addition of cations or by increasing the ionic concentration of the suspending medium (HARRIS, 1951). The age of the bacteria and the nutrient composition of the media influence of the surface charge of bacteria (PLUMMER and JAMES, 1961; MOYER, 1936; HILL et al., 1963); however, this does not occur for all organisms (JAMES, 1957).

(ii) Aggregation studies

Essentially two basic modes of bacterial aggregation have been described. One involves interaction between bacteria due to substances which are an integral part of the bacterial surface; the other involves high molecular weight polymers binding to and altering the nature of the bacterial surface or acting as a polymeric bridge between bacteria.

In many bacteria, factors favouring their aggregation usually involve a diminution of the electrical potential of the bacterial surface. By themselves, many bacteria will aggregate in the region of their iso-electric points where the net surface charge is least (LASSEUR et al., 1934; RYAN and KOLIN, 1964). SILVERMAN and KLEINBERG (1967) have shown that factors reducing the surface charge of the mixed bacterial population found in dental plaque also favour aggregation. They showed that lowering the pH, and raising the ionic strength or the concentration

of divalent cations increased bacterial aggregation.

Several studies have examined the use of macromolecular polyelectrolytes as bacterial flocculants (BUSCH and STUMM, 1968; GASNER and WANG, 1970). In most cases, satisfactory flocculation of bacteria with anionic polyelectrolytes requires the presence of calcium ions (NAKAMURA, 1961; BUSCH and STUMM, 1968). Studies of the ability of salivary derived polymers to flocculate oral bacteria have also revealed a dependency on the presence of calcium ions. Of eleven high molecular weight fractions isolated from plaque only those which contained high levels of calcium favoured flocculation of the plaque bacteria (SILVERMAN and KLEINBERG, 1967 b). Removal of calcium from saliva prevents the aggregation of oral bacteria by salivary polymers (GIBBONS and SPINELL, 1969; KASHKET and DONALDSON, 1972). According to SILVERMAN and KLEINBERG (1967 b) and BUSCH and STUMM (1968) the main role of divalent cations is ion pair formation or bridging between the functional groups of the polyelectrolyte and the bacterial surface, thus enhancing the adsorption of polymer segments.

(iii) Adherence studies

Adhesion of bacteria to surfaces may be divided into two stages; the first would be a reversible attachment dependent upon the nature of the bacterial and solid surfaces. The second would be an irreversible attachment dependent upon the substances produced by the attached bacteria during their metabolism (ZOBELL, 1943; MEADOW, 1971; MARSHALL et al., 1971).

In studies on the adsorption of marine bacteria to glass, MARSHALL et al., (1971) found that during the reversible phase, an increase in ionic strength or the presence of divalent cations favoured bacterial adsorption.

Adherence of oral microorganisms to various surfaces in the oral cavity has also been demonstrated. Of the many bacteria examined by HILLMAN et al., (1970) nearly all could adhere to either powdered human enamel of enamel pretreated with saliva. However, large differences in the degree of adsorption existed amongst the various bacteria. When coccobacillus strain 26 and Strep. mutans (strain 6715) were tested for adherence between pH 7.5 and 4.5, the adherence to untreated enamel increased with decrease in the pH. This could have been due to (i) minimization of the charged groups on the bacteria (ii) release of calcium from the hydroxyapatite of enamel and the calcium then enhancing enamel-bacterial interactions and (iii) both effects together with the phosphate of the suspending medium favouring a negatively charged enamel surface (NEIDERS et al., 1970). Variation of the pH had less effect on bacterial adherence when the enamel had been treated with saliva and thus covered with salivary polymers.

Van HOUTE et al., (1970, 1971) and GIBBONS and Van HOUTE (1971) examined the ability in humans of certain strains of oral streptococci either naturally present in the mouth or grown in vitro and introduced into the mouth as mixtures to adhere to a cleaned tooth surface, dental plaque or oral epithelium. The tooth surface and the surface covered with plaque favoured the accumulation of Strep. sanguis over Strep. salivarius. On the epithelium of the cheek and tongue both Strep. salivarius and Strep. sanguis accumulated more rapidly than Strep. mutans. In all of these studies, strain variability within the species examined was evident.

(iv) Physico-chemical forces involved in bacterial aggregation and adherence

It is apparent that the nature of the bacterial surface is an

important parameter of any of the interactions considered above. Since the surfaces of the cells carry a net negative charge and because under certain conditions aggregation and adsorption phenomena are favoured by increasing ionic strength and divalent cations, contact interactions of cells have been often considered in terms of the Derjaguin-Landau and Verwey-Overbeck (DLVO) theory (WEISS, 1970). This theory involves an estimation of the magnitude and variation with interparticle distance of the London-van der Vaals attractive energies and the electrical repulsive energies resulting from the overlapping ionic atmospheres (diffuse double-layers) around the surfaces.

With regard to electrostatic repulsive forces, cell surfaces bear charged groups which set up an electrostatic force which tends to repel charges (cells) of like sign. An electrical double layer covers a charged surface; on the inside it is made up of the charged groups of the surface and on the outside of the charged groups of opposite sign, the counter-ions. The thickness of the double layer is dependent on the electrolyte concentration and valency of the counter ions. Surfaces begin to interact appreciably when their double-layers overlap. The surface potential falls as the ionic concentration increases and divalent ions are usually more effective in lowering the potential than nonvalent ions. The repulsive forces are approximately equal to the square of the surface potential. This repulsive force forms a potential energy barrier which tends to prevent surfaces from coming closer together.

With regard to London-van der Waals forces, these are considered to be the main attractive ones between colloidal particles. The most important force is the London dispersion force due to the fact that in a neutral atom the zero point of energy of the electrons generates a fluctuating dipole which polarizes the surrounding atoms so they attract

one another. The London forces and the electrostatic forces of repulsion tend to balance one another at certain interface distances which are dependent upon the composition of the medium.

Purpose and Outline of this Thesis

The dental plaque, which forms spontaneously on the hard and soft oral tissues, produces large changes in pH which have been related to both caries and periodontal disease (KLEINBERG and JENKINS, 1964; FROSTELL and SODER, 1970; KLEINBERG, 1970 c). However, the cellular processes involved in the formation of the acid or base responsible for these pH changes are not clearly understood. The major reasons for this lack of information are the technical difficulties involved in monitoring plaque biochemical changes *in situ* and the availability of only small amounts of plaque material for *in vitro* investigation (KLEINBERG, 1970 a).

Pure cultures of plaque microorganisms have been used as models for studying plaque metabolism but such systems have several intrinsic difficulties. Firstly, the metabolism of a pure culture is not likely to be representative of the total metabolism of the plaque mixed microflora. Secondly, the procedures employed in isolating pure cultures may alter the enzymic compositions of the bacteria (HOMMES, 1965). Finally, bacteria which are members of mixed populations, such as those that occur on the hard and soft tissues of the human oral cavity, are subject not only to the chemical and physical influences of the host environment, but also to those of the other microorganisms within the population (STEPHAN and HEMMENS, 1947; RITZ, 1969; PARKER and CREAMER, 1971; REGOLATI *et al.*, 1972). It is for these reasons that studies of pure

cultures must ultimately be extended to the actual mixed cultures characteristic of the bacterial populations in the oral cavity. To aid in overcoming these problems KLEINBERG (1967 a) developed an *in vitro* model from the sediment of wax-stimulated whole saliva, which produces pH curves similar to those observed in plaque *in situ*.

Subsequent studies have described many of the processes involved in the carbohydrate and nitrogen metabolism of the sediment mixed microbial flora which account for these pH curves (for a review see KLEINBERG, 1970 a). It has not been fully established, however, if dental plaque *in vitro*, under the same cell concentration and substrate conditions, behaves in a similar manner. Since plaque *in situ* possesses certain structural characteristics and environmental influences which are difficult to duplicate *in vitro*, experiments were carried out to determine whether metabolic processes demonstrated in sediment also occur in plaque *in situ*. Since acid-base changes do not occur on the tooth surface without the plaque, a study was also carried out in which some physico-chemical factors important in the aggregation behaviour of the plaque bacteria and which may be important in the formation of plaque *in situ* were examined.

In the first study of this thesis, the suspension concentrations of plaque and salivary sediment which yield similar pH time curves when incubated with glucose were determined. Once these conditions were established, experiments were carried out to test the effects of other substrates and substances in both plaque and sediment incubation mixtures at these matched cell concentrations (CHAPTER II). The second study examined the ammonia and urea levels in maxillary and mandibular incisor plaques and compared these to the pH levels previously found to be characteristic for these sites (CHAPTER III). The uptake and clearance of urea and glucose from plaque and saliva following a rinse with either

urea or glucose and the formation and disappearance of ammonia in plaque following a rinse with urea were examined in the third study (CHAPTER IV). The fourth study dealt with the composition of the free amino acid pools of fasted plaques located on different incisor surfaces. Once this was established the effects of an oral rinse with either urea, glucose, urea plus glucose or distilled water on the composition and size of such pools was investigated (CHAPTER V). In the final study of this thesis, the effect of a number of physico-chemical factors on the aggregation behaviour of pure and binary mixtures of various oral streptococci was examined. Aggregation of mixed populations of these bacteria was then explored. A partial separation of the bacteria in plaque was then accomplished using the technique of continuous particle electrophoresis. Aggregation of these bacterial fractions and the mixed populations obtained upon culturing plaque from the enamel and gingivae was also examined (CHAPTER VI).

Methods used in this thesis are described in the methods section of the various chapters.

A summary and statement of the conclusions in CHAPTER VII complete the thesis.

CHAPTER II

A COMPARISON OF THE EFFECTS OF VARIOUS FACTORS UPON THE pH OF DENTAL PLAQUE AND SALIVARY SEDIMENT INCUBATION MIXTURES.

The pH of the dental plaque *in situ* rapidly falls, reaches a minimum and then slowly rises following a glucose or sucrose rinse (STEPHAN, 1940). Further, the minimum pH obtained is inversely related to the caries activity (STEPHAN, 1944). Using a system containing pure cultures of plaque bacteria suspended in a medium of inorganic salts, STEPHAN and HEMMENS (1947) demonstrated that the fall-rise shape of the Stephan pH curve occurs only when the bacterial cell concentration is high and the glucose concentration is low. Similar results have been obtained by STRALFORS (1950) using a glass pH electrode encased by an agar gel within which a pure culture of an acidogenic plaque micro-organism is embedded.

The Stephan type of pH curve has been demonstrated with the mixed bacterial populations found in wax-stimulated whole saliva when incubated with glucose in either suspended (KLEINBERG, 1967a) or packed form (MANLY *et al*, 1962). A reverse Stephan curve (i.e., a rapid rise followed by a slow fall) occurs in plaque *in situ* when urea replaces fermentable carbohydrate as substrate (KLEINBERG, 1967b). This has also been observed with salivary sediment under high and low substrate conditions (KLEINBERG, 1970a; BISWAS and KLEINBERG, 1971).

The appearance of the slow rise after the pH minimum with glucose and the slow fall after the pH maximum with urea (that is, the return of the pH to starting pH levels) can be delayed both in

plaque *in situ* (KLEINBERG, 1961 and 1967 a, b) and in the suspended salivary sediment (SSS) system *in vitro* by prolonging the period that substrate is available (KLEINBERG, 1970a).

Apparent from the above experiments is the fact that concentration and substrate availability are basic deteriminants of the pH changes seen in the different bacterial systems.

Using glucose and urea as substrates, the carbohydrate and nitrogen metabolisms of the suspended salivary sediment (SSS) system have been extensively studied in relation to changes in pH; these changes were similar to those previously observed in plaque *in situ* (KLEINBERG, 1967a, b). Because sediment and plaque show certain differences in incidence of *Strep. mitis* and *Strep. salivarius* and differences in calcium and phosphate content, it has been suggested that sediment may not be a suitable model for the study of plaque metabolism (KRASSE, 1954; GIBBONS, *et al*, 1964; DAWES AND JENKINS, 1962). In order to test the significance of such differences, the present study examines whether or not each of a number of factors, shown previously to affect the metabolism and the pH response of the SSS system, has a similar effect on the pH and accordingly the metabolism of the plaque microflora.

In the first series of experiments, both the suspension and the glucose concentrations were varied in plaque and salivary sediment incubation mixtures to establish the cell and substrate conditions under which the two systems have the closest glycolytic activities and produce similar pH curves. This examination was carried out in the presence and absence of salivary supernatant, since this is the medium of both the salivary sediment and dental

plaque microfloras and has been shown to have a marked influence on their metabolisms (KLEINBERG et al. 1973; KORAYEM and KLEINBERG, 1973). Once accomplished, factors shown previously to have substantial effects on the pH of incubated salivary sediment mixtures were examined under similar conditions for their effects on the plaque microflora. These were: L(+) and D(-) lactic acid utilization, (SANDHAM and KLEINBERG, 1969a; KOMIYAMA and KLEINBERG, 1973), urea catabolism (BISWAS and KLEINBERG, 1969b), the addition of a salivary fraction containing a pH-rise factor (PRF) which stimulates glycolysis (KLEINBERG et al., 1968) and the addition of fluoride which inhibits glycolysis (SANDHAM and KLEINBERG, 1969b).

The buffering capacities of the plaque and salivary sediment mixtures with the cell concentrations selected were also compared to determine what influence, if any, buffering had on the pH response. This was done both in the presence and the absence of salivary supernatant.

Methods

Preparation of plaque and salivary sediment incubation mixtures

Plaque (25-100 mg wet weight) was collected on the morning of the fourth day from 1 to 5 subjects who had not cleaned their teeth for 3 days nor eaten within the 12 hr period prior to plaque collection (KLEINBERG and JENKINS, 1964). The plaque, upon removal with a stainless steel spatula, was immediately dispersed in 250 µl of ice-cold distilled water. After pooling, the plaque was centrifuged (1740 g for 15 min at 4°C), the supernatant discarded and the pellet then washed 3 times with cold distilled water before re-suspending in

distilled water at concentrations of 5, 25 or 50 per cent (V/V). Wax-stimulated whole saliva (0.5 to 1 ml) was then collected from each subject in a test tube previously chilled in cracked ice. The saliva samples were pooled, centrifuged and the sediment washed in the same manner and suspended at the same suspension concentrations as the plaque (cf KLEINBERG, 1967a). All preparative procedures were carried out in a cold room at 4°C.

Plaque and salivary sediment mixtures (60 µl) were prepared in cup-shaped glass pH microelectrodes (G221C, Radiometer, Copenhagen) supported within an acrylic frame (Fig. 2.1). Both the frame and stoppers were necessary to minimize moisture loss and contamination of the mixtures with atmospheric carbon dioxide or ammonia, either of which would alter the pH.

The mixtures had the following compositions and final concentrations: (i) cells - plaque or sediment suspended at either 1.7, 8.3 or 16.7 per cent (V/V); (ii) medium - either salivary supernatant at 33.3 per cent (V/V), a salivary fraction containing pH-rise factor (see below) at 3 times the concentration found in supernatant, or distilled water; (iii) substrate - glucose at either 0.0, 0.05, 0.1 or 0.5 per cent (W/V), L(+) or D(-) lactic acid at 0.1 per cent (W/V), or urea at 0.033 or 0.17 per cent; and (iv) in some experiments with glucose, either 0 or 5 ppm fluoride as the sodium salt.

Incubation procedures

The pH was measured at the onset and at intervals during each incubation (4 hr at 37°C). To measure the pH the rubber stopper covering the opening in the chamber above each electrode was removed, the contents of the cup was stirred with a fine tapered teflon rod with a slight curve at its fine end (0.5 mm. diam) and then a fine

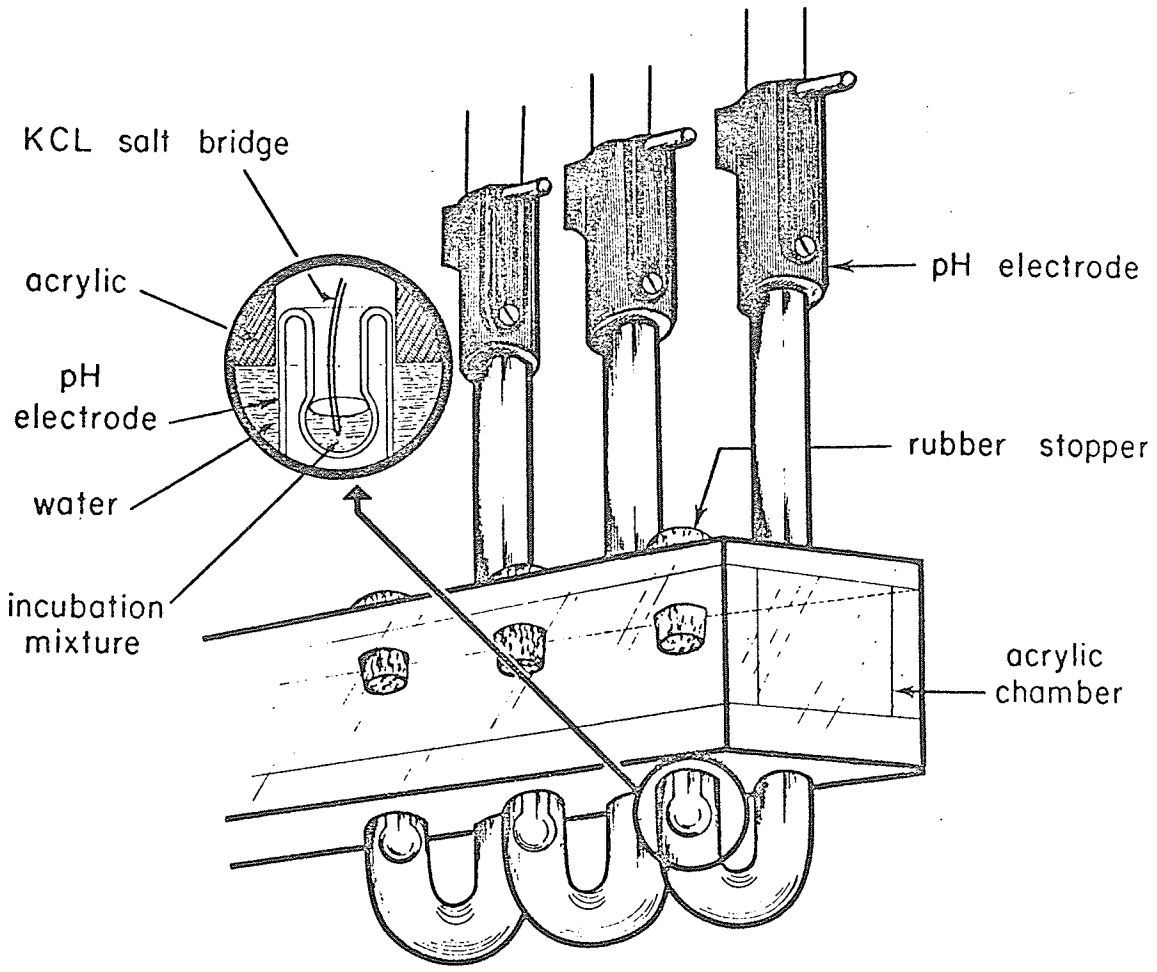


Fig. 2.1. Schematic diagram of the chamber and the cup-shaped pH micro-electrodes used for the plaque and sediment incubation mixtures.

KCl salt bridge (KLEINBERG, 1967a) leading from a calomel electrode was inserted into the mixture and the pH read (see insert Fig. 2.1) The salt bridge was immediately removed and the stopper replaced.

The KCL salt bridges all led to one calomel reference electrode. It and the 6 pH electrodes were connected through a 6 channel switching device (model SAS 1 Radiometer, Copenhagen) to a pH meter (Type PHM 26 Radiometer, Copenhagen).

(a) Effect of glucose concentration and salivary supernatant on the pH of plaque and sediment suspensions at adjusted cell concentrations

In each experiment 4 mixtures were prepared, 2 containing plaque and 2 containing sediment. One sediment and one plaque mixture contained salivary supernatant; the other two mixtures were their distilled water controls. One cell concentration [1.7, 8.3 or 16.7% (V/V)] was treated in each experiment with one glucose concentration [0.1 or 0.5 per cent (W/V)]. This design results in 6 different experiments, each being carried out at least twice.

(b) The effect of salivary PRF fraction, lactic acid, urea and fluoride upon the pH of plaque and sediment mixtures at adjusted cell concentrations

The experiments in section (a) showed that pH curves were most similar when plaque and salivary sediment concentrations were 8.3 and 16.7 per cent (V/V), respectively. Therefore, these suspension concentrations were selected for examination and comparison of the following variables on plaque and sediment pH: (i) either a salivary PRF fraction, salivary supernatant at 33.3 per cent (V/V) or distilled water incubated with glucose at 0.05 per cent (W/V); (ii) L(+) or D(-) lactic acid at 0.1 per cent (W/V) incubated in the presence of salivary supernatant; (iii) urea at either 0.033 or 0.17 per cent (W/V) with and without supernatant; and (iv) fluoride at 5 ppm incubated with glucose at either

0.05, 0.1 or 0.5 percent (W/V) with supernatant present.

In each experiment, 4 mixtures were incubated at the same time; two contained plaque, the other two contained salivary sediment. Thus, several experimental trials were required to carry out each of the above experiments and all incubations were carried out at least twice.

Buffering capacities of salivary sediment and plaque-saliva mixtures

Plaque mixtures (50 ul) at a suspension concentration of 8.3 percent (V/V) and sediment mixtures (50 µl) at a suspension concentration of 16.7 percent (V/V) were titrated between pH 4 and 10 with 0.5 N HCl; this was done both in the presence and absence of salivary supernatant. Each mixture was prepared and titrated at room temperature within a cup-shaped pH microelectrode.

In order to add the very small aliquots of acid required, a wire loop was fabricated from stainless steel wire (0.007 in diam) and calibrated as follows. Solutions containing 3 different levels of inorganic orthophosphate were prepared in quadruplicate by transferring 2, 3 and 4 aliquots of a 7.0 M K_2HPO_4 (1.25 ugP/ul) solution into test tubes containing 200 ul of distilled water. The phosphate in each of the 12 samples was analyzed in triplicate by the method of KUTTNER and COHEN (1927). It was found that the loop transferred 0.121 ul of fluid (coefficient of variation = 7.5 percent).

The loop was used in each titration as follows. The loop was dipped into the acid and then inserted into the plaque or sediment mixture; it was rapidly rotated between the thumb and forefinger to ensure complete transfer and dispersion of the acid in the incubation mixture. Between each acid addition, the wire loop was washed in a stream of

distilled water and dried by blotting with tissue paper.

Results

(a) Effect of glucose and salivary supernatant on the pH of plaque and sediment mixtures at unmatched and matched suspension concentration.

Whether or not supernatant was present, and with either 0.1 or 0.5 percent glucose as substrate, the pH fell faster as the cell concentration of either the sediment or plaque was increased (FIGS. 2.2 and 2.3).

With 0.1 percent glucose, a faster pH fall was associated generally with a tendency for the pH to reach an earlier minimum and show an earlier rise. In some cases, a slower pH fall and a slower pH rise with sediment resulted in a lower pH being reached than with plaque. In other cases, the sediment pH minimum was higher than for plaque.

With 0.5 percent glucose, a pH rise was not seen with sediment but a slight pH rise did occur in the plaque mixture having the highest suspension concentration.

From inspection of the various pH curves obtained in these experiments it would appear that plaque at 8.3 percent yields pH curves quite similar to sediment at 16.7 percent (FIG. 2.4). This similarity was most marked when supernatant was present. Interestingly, the pH fall was less and the subsequent pH rise occurred sooner with plaque than with sediment when both were incubated in the absence of both glucose and supernatant.

(b) The effects of salivary pH-rise factor, fluoride, D(-) and L(+) lactic and urea upon the pH of plaque and sediment mixtures at cell concentrations of 8.3 and 16.7 percent (V/V) respectively.

(i) salivary pH-rise factor

When salivary supernatant was replaced with a salivary PRF

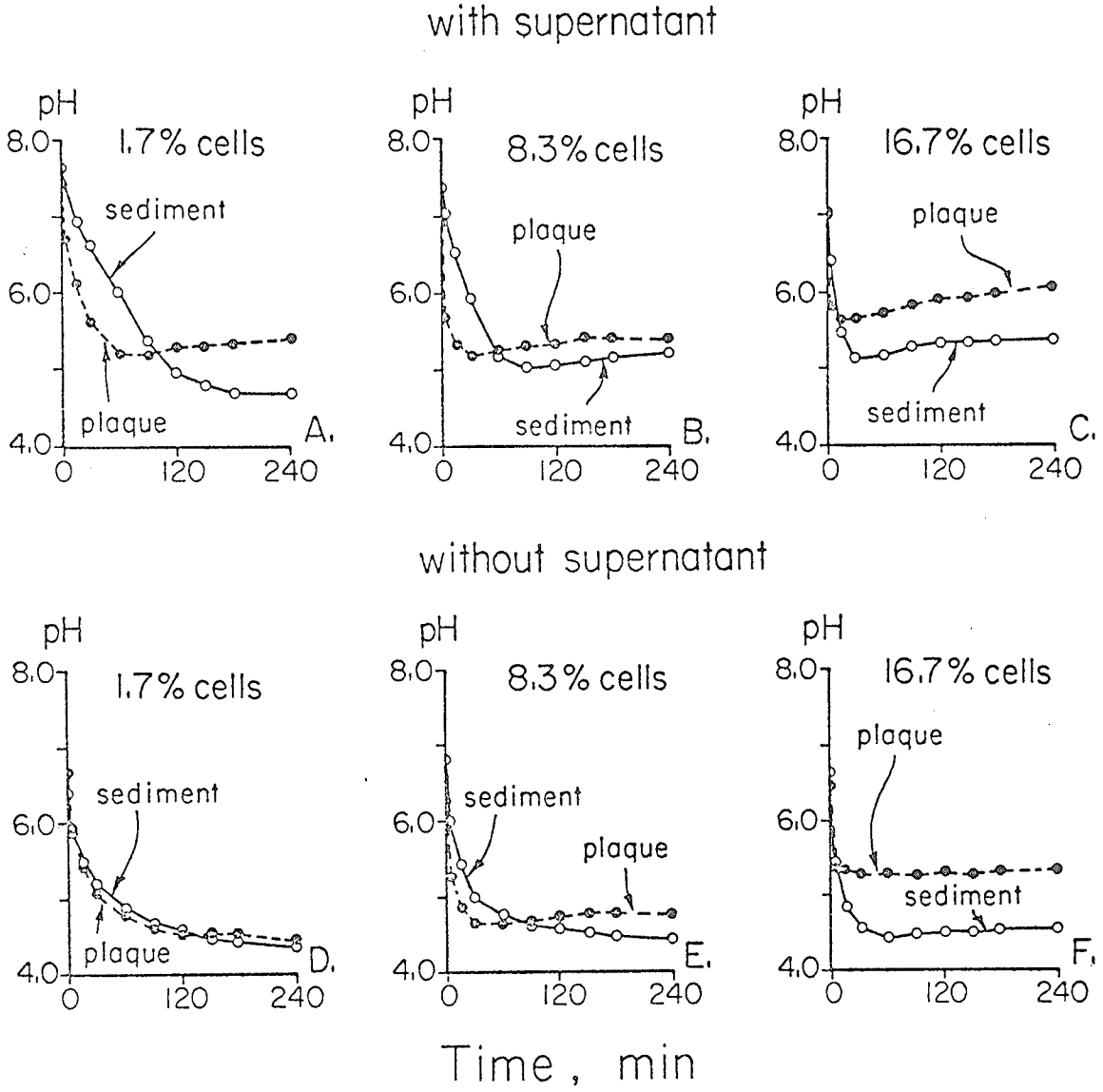


Fig. 2.2. The effect of suspension concentration (1.7, 8.3 and 16.7 per cent (V/V)) on the pH of plaque and salivary sediment mixtures incubated with 0.1 percent (W/V) glucose, either in the presence or the absence of salivary supernatant (33.3 per cent (V/V)).

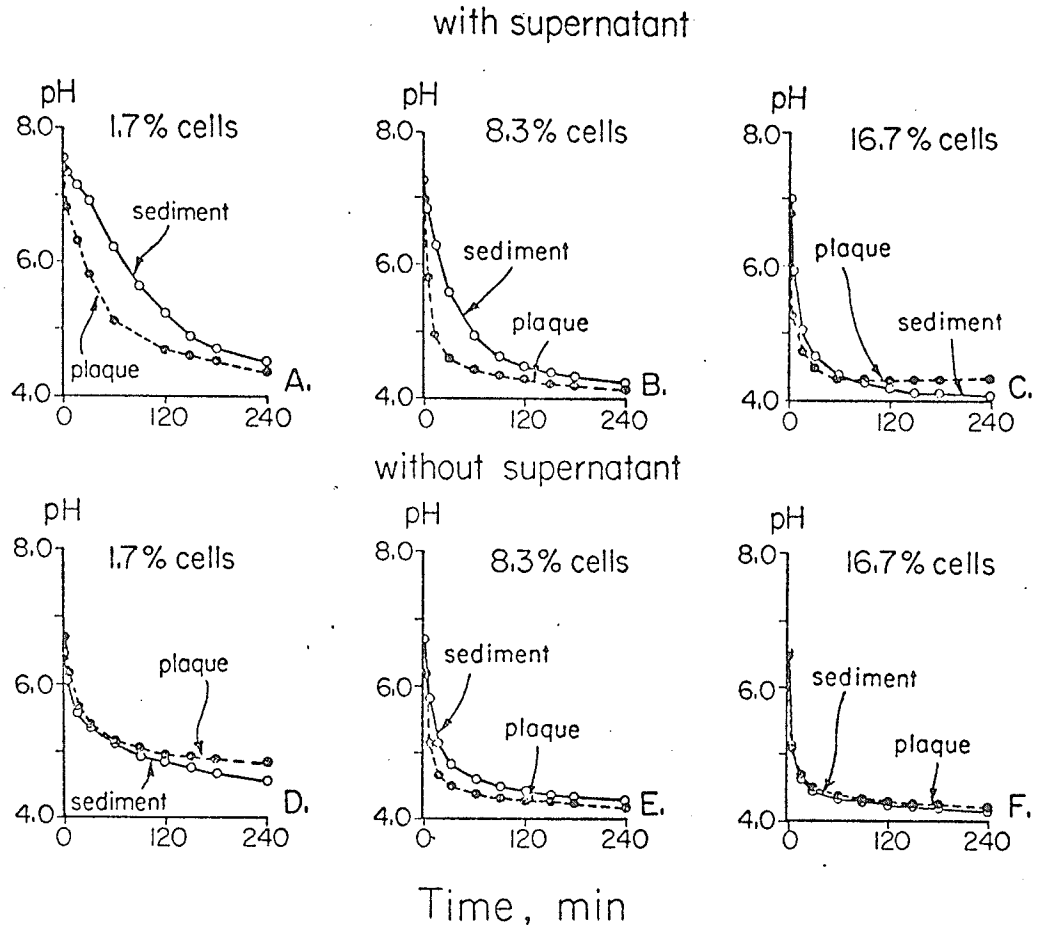


Fig. 2.3 The effect of suspension concentration (1.7, 8.3, and 16.7 percent (V/V)) on the pH of plaque and salivary sediment mixtures incubated with 0.5 percent (W/V) glucose, either in the presence or the absence of salivary supernatant (33.3 percent (V/V)).

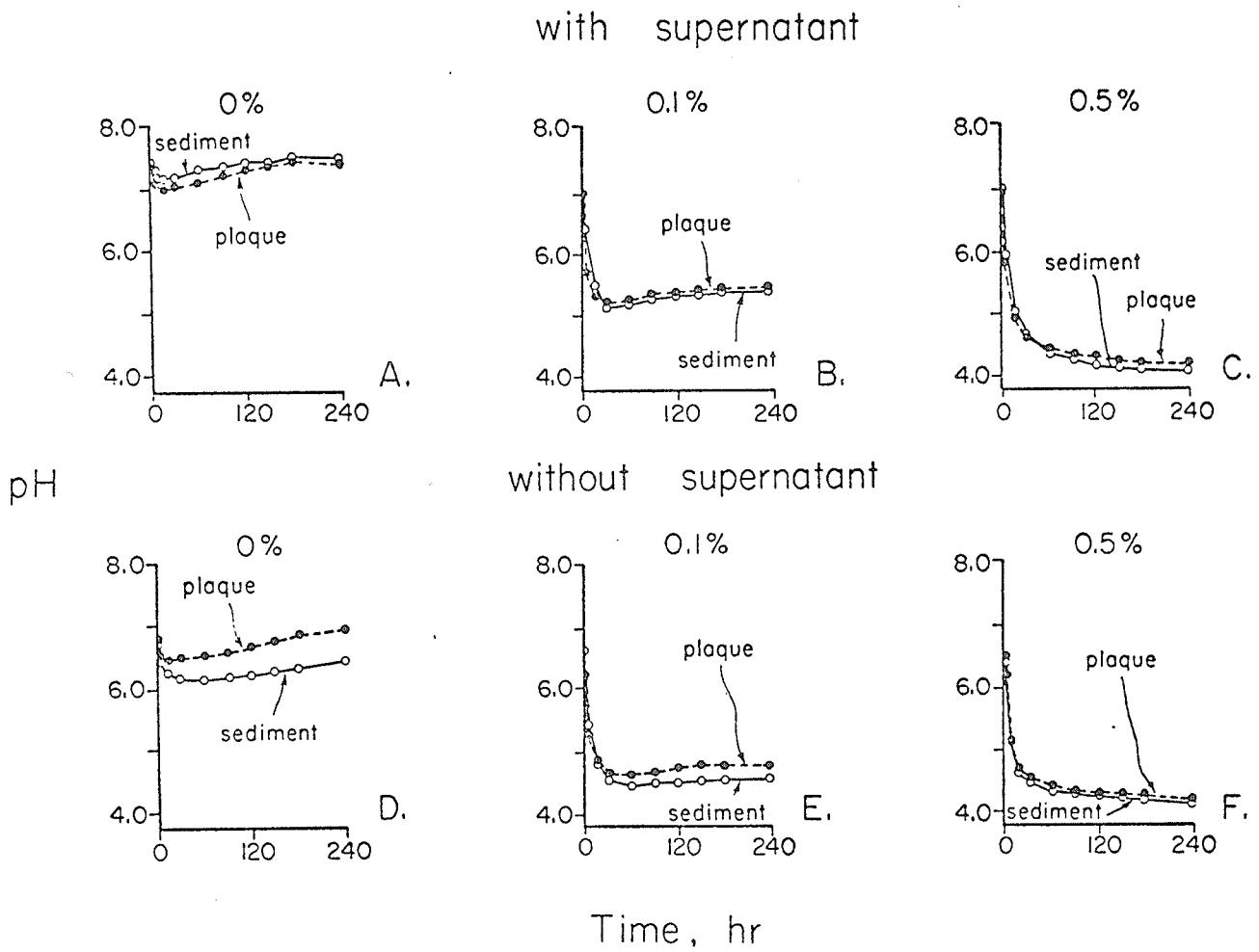


Fig. 2.4. Comparison between the pH changes in mixtures of 8.3 percent (V/V) plaque and 16.7 percent (V/V) salivary sediment when incubated with glucose (0, 0.1 or 0.5 percent (W/V)), either in the presence or absence of salivary supernatant (33.3 percent (V/V)).

fraction, the pH of both plaque and sediment mixtures behaved as if salivary supernatant was present (FIG. 2.5); the pH-rise began earlier in both. Since both supernatant and pH-rise factor have a slightly basic pH, the pH of the control mixtures were adjusted at the onset with 5mM NaOH to ensure that the initial pH was similar to the experiment mixtures. This adjustment had no obvious effect on the pH response in these experiments with either supernatant or the PRF fraction.

(ii) fluoride

The effects of 5 ppm fluoride on the pH of both plaque and sediment mixtures was the same. Inhibition in both cases was dependent upon the glucose concentration (FIG. 2.6) (cf Sandham and Kleinberg, 1969b). With 0.05 percent glucose, fluoride stimulated the pH fall slightly, whereas, with 0.5 percent glucose, fluoride had a marked inhibitory effect. With 0.1 percent glucose, no effect of fluoride was noticed.

(iii) D(-) and L(+) lactic acid

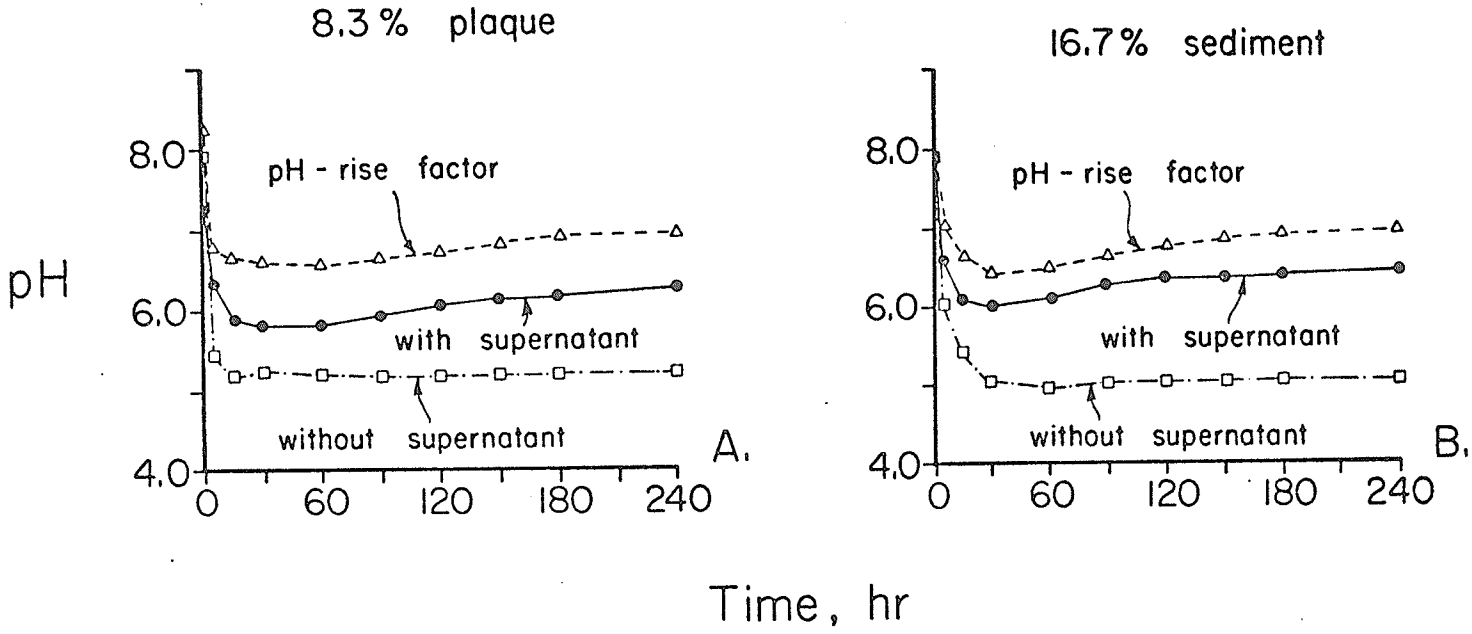
As demonstrated earlier with salivary sediment (SANDHAM and KLEINBERG, 1969a; KOMIYAMA and KLEINBERG, 1973), the pH rose as D(-) or L(+) lactic acid was utilized by the plaque bacteria FIG. 2.7).

(iv) Urea

With 0.17 and 0.033 percent urea (FIG. 2.8), the pH rose and fell in both the plaque and the sediment mixtures whether or not supernatant was present; however, the rates were faster in both cases with plaque.

(c) Comparison of the buffering capacity of 8.3 percent plaque and 16.7 percent sediment suspensions

Titrations of plaque and sediment mixtures were almost identical



2.5. Comparison between the pH changes of plaque (8.3 percent (V/V)) and salivary sediment (16.7 percent (V/V)) mixtures when incubated with 0.05 percent (W/V) glucose, either in the presence of salivary supernatant (33.3 percent (V/V)), a salivary fraction containing pH-rise factor (activity 3 1/3 times that in whole saliva) or distilled water.

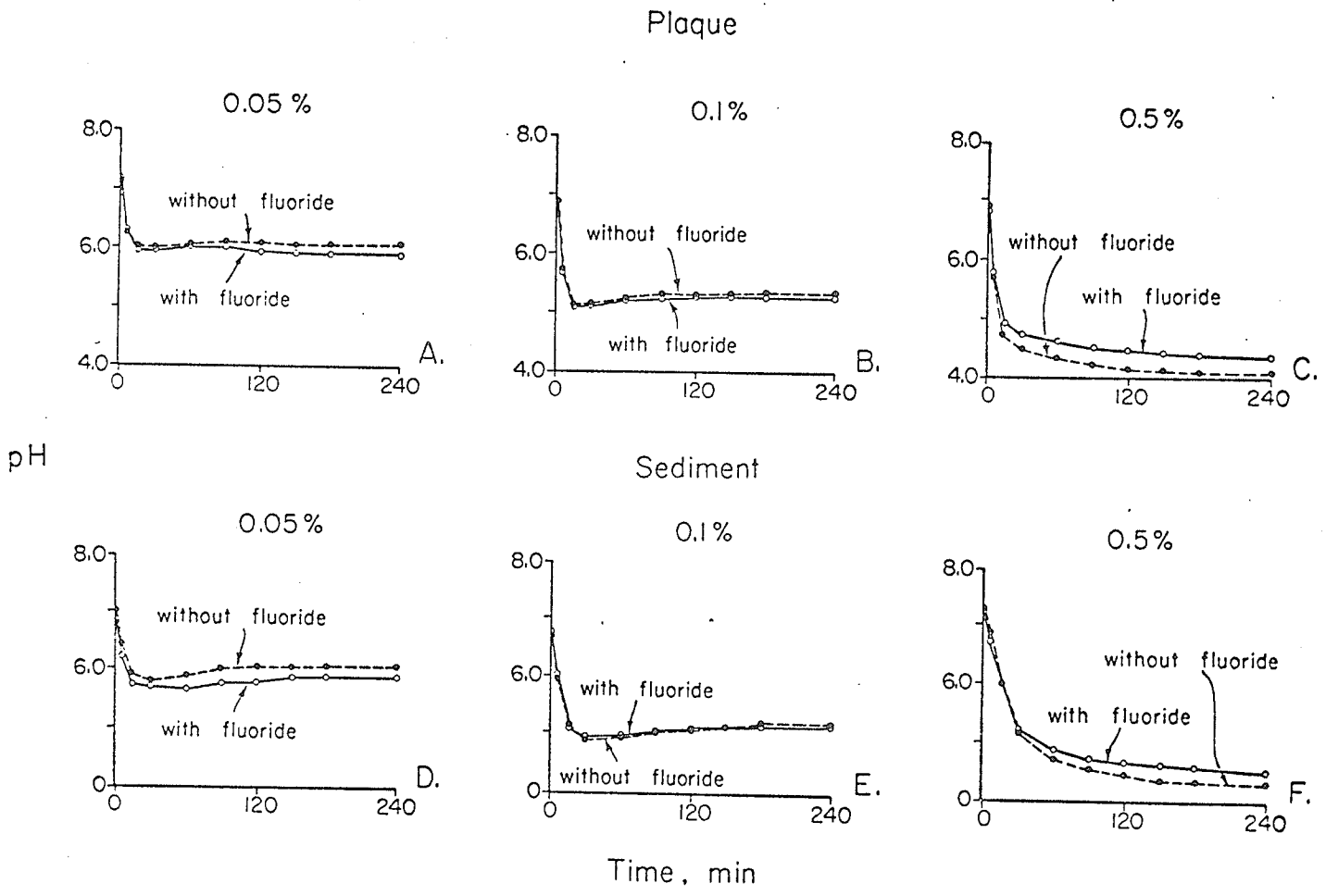


Fig. 2.6. The effect of fluoride on the pH of plaque (8.3 percent (V/V)) and salivary sediment (16.7 percent (V/V)) mixtures when incubated with glucose at 0.05, 0.1 or 0.5 percent (W/V) and in the presence of salivary supernatant (33.3 percent (V/V)).

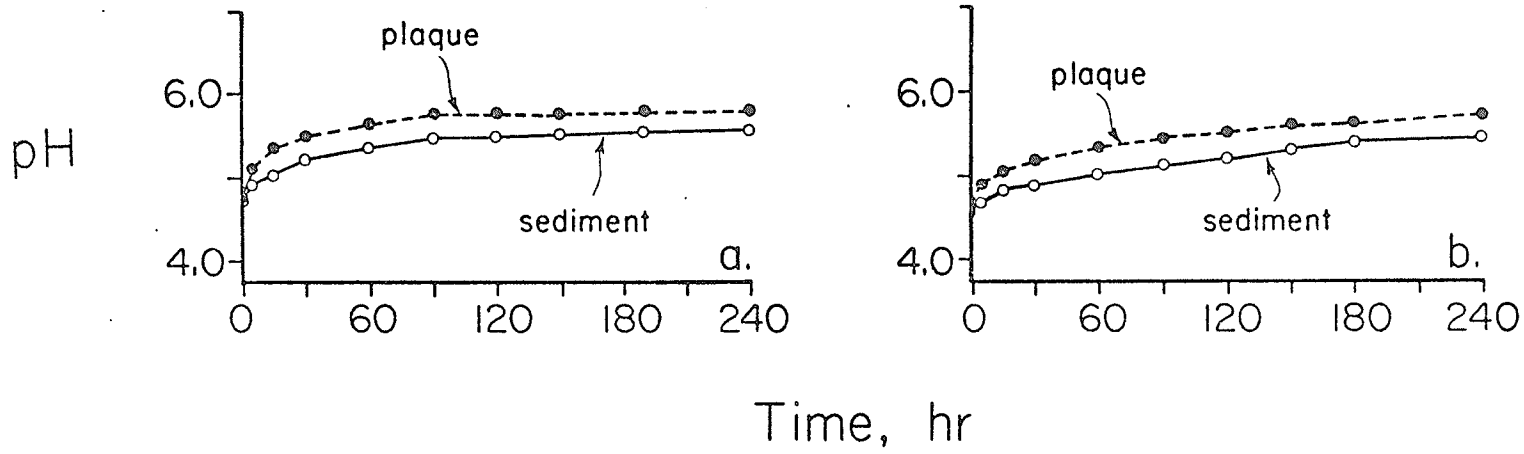


Fig. 2.7. Effect of (A) L(+) lactic acid (0.1 percent (W/V)) and (B) D(-) lactic acid (0.1 percent (W/V)) on the pH changes of plaque (8.3 percent (V/V)) and sediment (16.7 percent (V/V)) mixtures when incubated in the presence of salivary supernatant (33.3 percent (V/V)).

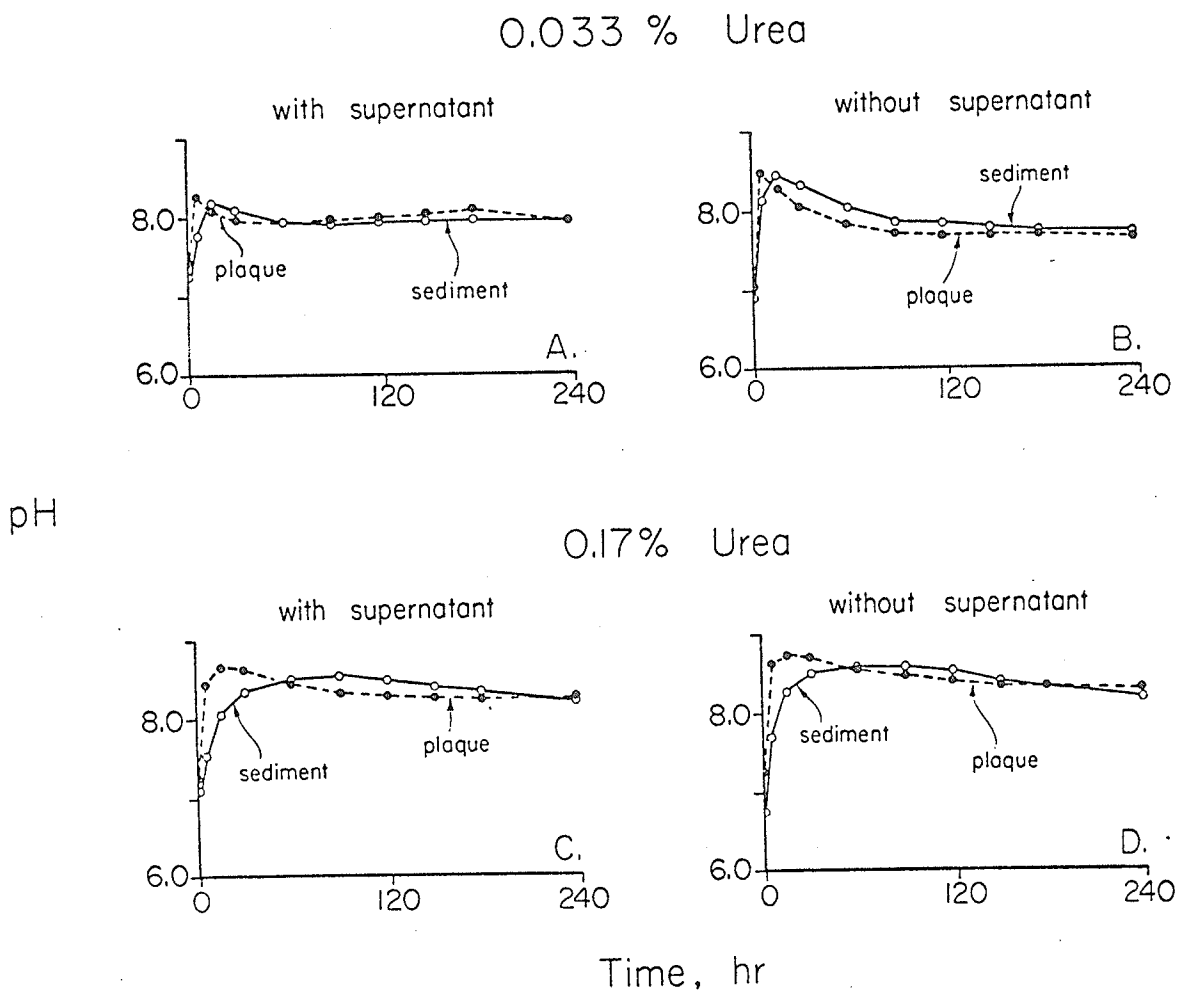


Fig. 2.8. Comparison between the pH changes of plaque (8.3 percent (V/V)) and salivary sediment (16.7 percent (V/V)) mixtures when incubated with urea (either 0.033 percent or 0.17 percent (W/V)) in the presence of salivary supernatant (33.3 percent (V/V)) or distilled water.

over the pH range examined (4 to 10) when salivary supernatant was present (Fig. 2.9). However, the buffering capacities of the plaque and sediment mixtures were both less in the absence of supernatant; the sediment mixtures showed the lower buffering capacity.

DISCUSSION

The results in the present study have demonstrated that the pH response of both the plaque and salivary sediment microfloras are similar when suspensions are incubated and compared under a variety of conditions. With both microfloras (i) supernatant in the presence of a low concentration of glucose inhibited the extent of the pH fall, stimulated an earlier occurrence of the pH minimum and an earlier onset of the subsequent rise in the pH; (Figs. 2.2, 2.4 and 2.5); (ii) the salivary PRF fraction under similar conditions gave similar results; (iii) supernatant stimulated the pH fall slightly with 0.5 percent glucose (Figs. 2.3 and 2.4); (iv) the pH rose with both D(-) and L(+) lactic acid and almost identical curves were observed (Fig. 2.7); (v) fluoride stimulated the pH fall with 0.05 percent glucose, had no effect with glucose at 0.1 percent but inhibited the pH fall with 0.5 percent glucose (Fig. 2.6) and (vi) urea showed a rise and a fall in the pH both in the presence and absence of supernatant (Fig. 2.8).

The only significant differences between the two systems were: (i) the plaque changes with urea were more rapid than the sediment changes (Fig. 2.8) and (ii) the pH fall was less and the return of the pH was sooner with plaque than with sediment when they were incubated in the absence of both supernatant and added substrate (Fig. 2.4).

These results along with those in earlier studies showing a

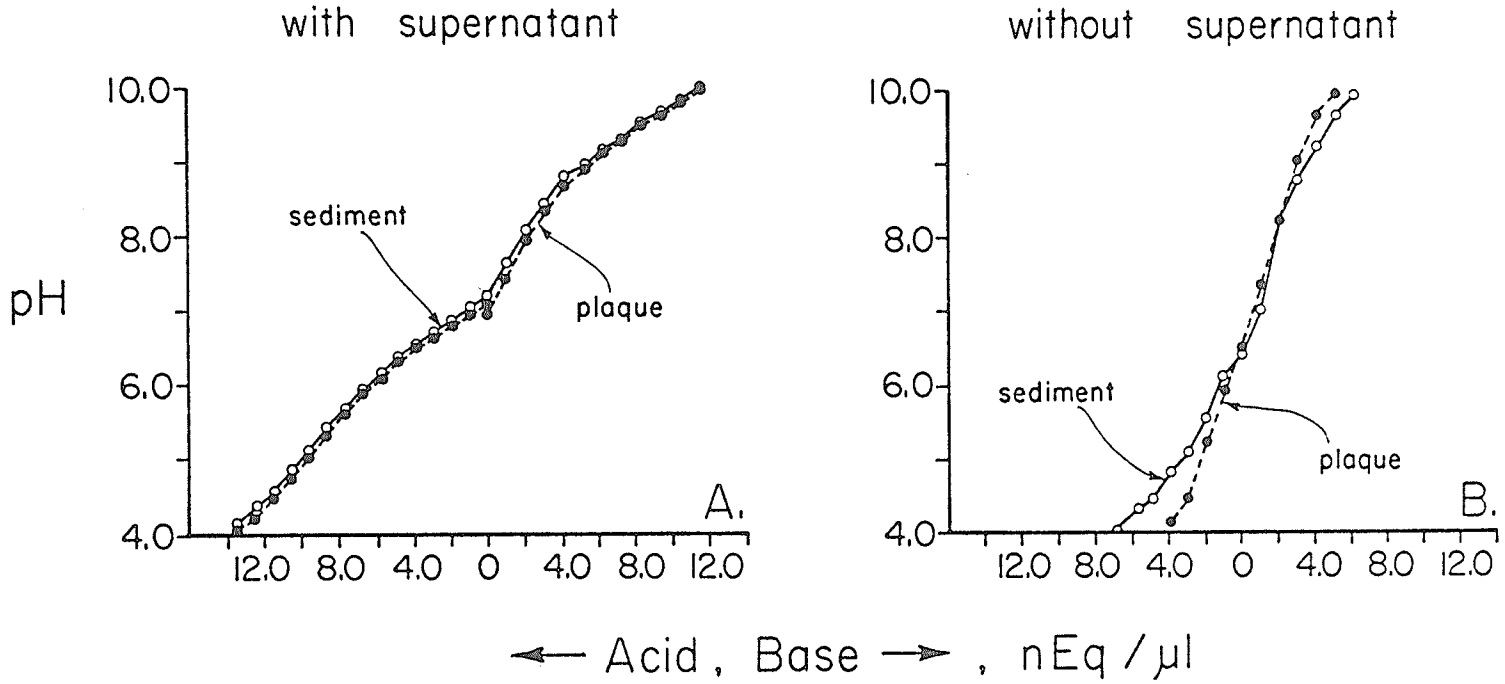


Fig. 2.9. The buffering capacity of 8.3 percent (V/V) plaque mixtures and 16.7 percent (V/V) sediment mixtures (A) in the presence of salivary supernatant (33.3 percent (V/V)) and (B), in the absence of salivary supernatant (distilled water). The titrant was either 0.1N HCl or 0.1N NaOH.

number of similarities in the metabolisms of the two systems (KORAYEM, 1973; HALHOUL, 1972; REDDY, 1973), provide strong evidence for the acid-base metabolisms of the two microbial systems being similar.

The greater activity of plaque than that of an equal volume of sediment may be due to several factors. Firstly, sediment contains more epithelial cells than plaque (TONZETICH and FRIEDMAN, 1965). Consequently, one could expect these cells to have a larger diluting effect on the sediment than on the plaque bacteria. The sediment, therefore, would show comparatively less metabolic activity per unit volume (KLEINBERG, 1967a; TATEVOSSIAN and JENKINS, 1969). Secondly, there are differences in the two systems. For example, salivary sediment appears to have a lower incidence of Strep. mitis and a higher incidence of Strep. salivarius than that reported for unclassified dental plaque (KRASSE, 1954; GIBBONS et al., 1964). This could result in differences in glycolytic activity. Finally, the buffering capacities of the two systems might differ.

The present study has shown that these bacterial differences are of little consequence if the pH activities are matched. This is probably because cell concentration is such a dominant factor that compensating for this variable is all that is necessary to observe similar metabolic responses in the two systems. Besides, the differences in incidence of Strep. mitis and Strep. salivarius are probably not significant since there seems to be very little difference in the types and amounts of acid formed from glucose amongst many of the oral streptococci (DRUCKER and MELVILLE, 1968).

With regard to buffering capacity, 8.3 percent plaque suspension and 16.7 percent sediment suspension showed similar capacities when supernatant was present; however, the buffering capacity of plaque was slightly

greater in the absence of supernatant (Fig. 2.9). The difference may be due to the presence in plaque of considerably higher levels of calcium phosphate than in salivary sediment (DAWES and JENKINS, 1962; KLEINBERG et al., 1971). The acid added to calcium phosphate is neutralized in the dissolution process (REDDY, 1973), hence the buffering effect. This difference in buffering between plaque and sediment suspensions could account for the poorer correlation between the pH curves of these mixtures in the absence of supernatant than in its presence. It may also account for the fact that in the absence of both supernatant and glucose, the pH falls less with plaque than with sediment (Fig. 2.4). The pH fall has been attributed to the degradation of the small amounts of stored carbohydrate in salivary sediment (SANDHAM and KLEINBERG, 1969a) which may also apply for dental plaque under these conditions. In the absence of the comparatively large buffering effect of supernatant, altering the suspension concentration would in addition to altering the ability of the microfloras to produce acid alter the buffering capacity somewhat (KLEINBERG, 1967a).

In addition to providing further support for acid-base metabolisms of plaque and salivary sediment microfloras being similar, the present investigation defines the experimental conditions needed for further comparative metabolic studies.

C H A P T E R I I I

AMMONIA AND UREA CONTENT OF DENTAL PLAQUES LOCATED IN DIFFERENT REGIONS OF HUMAN INCISORS

Before breakfast, the pH of the dental plaque is higher than that of the surrounding saliva (KLEINBERG and JENKINS, 1964). This difference has been attributed to the plaque bacteria forming ammonia from salivary urea faster than the ammonia can be neutralized by salivary buffers. In support of this explanation are the observations that: (i) urea degradation in either dental plaque or salivary sediment suspensions causes a simultaneous rise in both the ammonia concentration and the pH (FROSTELL, 1960; BISWAS and KLEINBERG, 1971); (ii) urea causes a rapid rise in the pH of dental plaque in situ; and (iii) both the extent and duration of the pH rise increase with increase in the amount of urea available (KLEINBERG, 1967 b).

Plaques in regions of the human dentition exposed to large amounts of saliva, such as the mandibular incisors, show a higher pH than plaques from the maxillary incisors where exposure to saliva is much less (KLEINBERG and JENKINS, 1964). Such regional variation in the pH has been attributed to variation in ammonia formation arising from differences in the availability of salivary urea.

In order to test the hypothesis that ammonia formation from salivary urea is mainly responsible for the high pH of fasting plaques and for the regional differences in the plaque pH, the present study has examined (i) whether or not urea and ammonia levels are higher in those plaques exhibiting a higher pH and (ii) whether ammonia could be the base mainly responsible for the differences in pH.

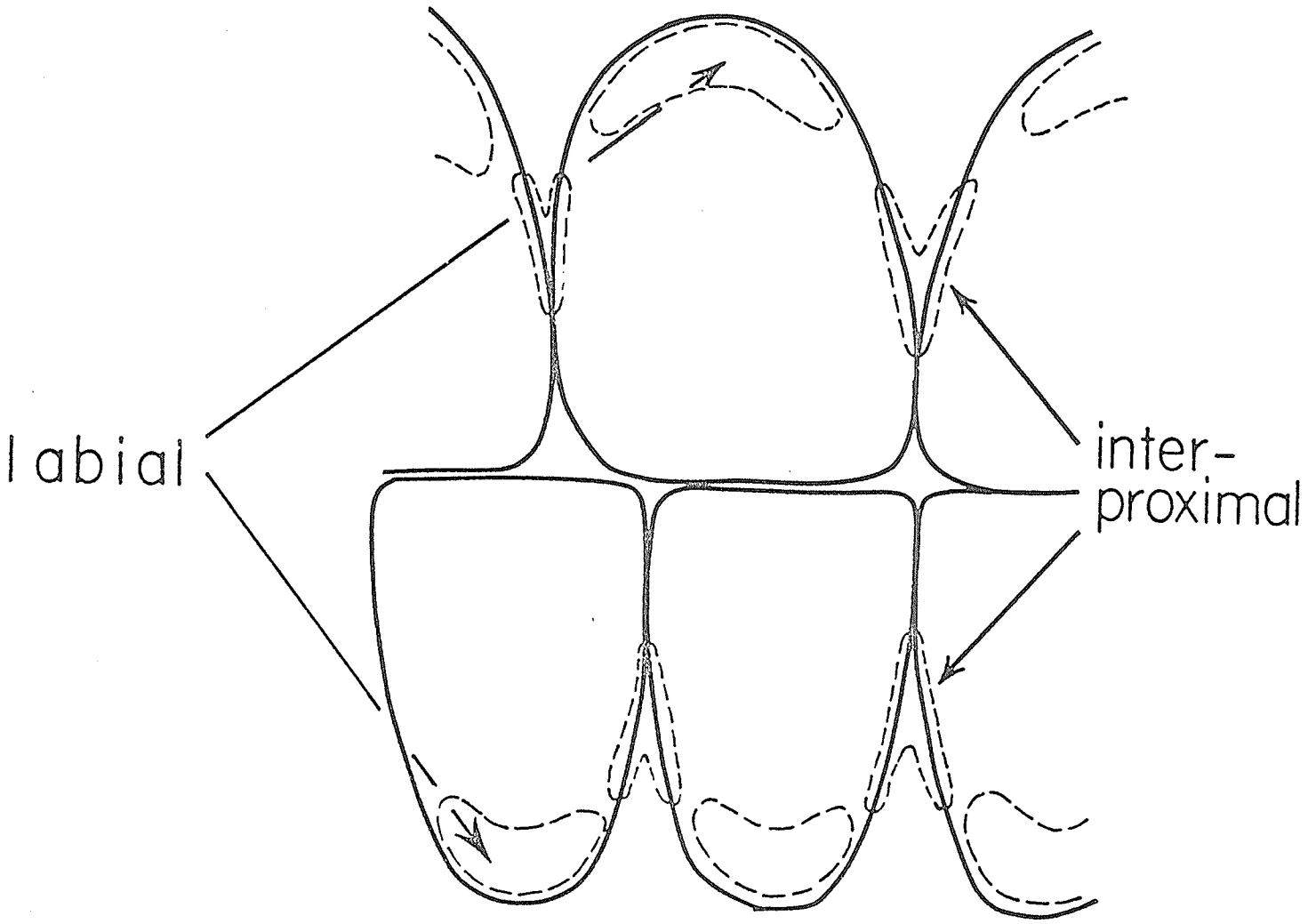


Fig. 3.1. Regions of the incisor teeth from which plaque was sampled.

METHODS

Plaque sampling and preparation for chemical analysis

Two labial and two interproximal samples of plaque (0.04 - 2.4 mgm wet weight) were removed from both the maxillary and mandibular incisors of each of nine individuals for ammonia and nitrogen analyses (Fig. 3.1). The latter analysis was for the purpose of measuring the plaque sample size (see section (a) below). The subjects were instructed not to clean their teeth for three days nor eat for at least 12 hours prior to the time of plaque collection, which occurred on the morning of the fourth day between 8:30 and 9:30 a.m. Before the plaque samples were taken, each subject sat quietly with head erect and lips closed for five to ten minutes. The samples were removed in random order with a stainless steel spatula at intervals of one to two minutes. Upon removal, each sample was immediately transferred to a polyethylene centrifuge tube (Beckman Model 152, Microfuge) containing 0.1 N HCl (25 μl) and chilled in cracked ice.

For the urea experiments, plaques were sampled from two groups of subjects. In the first group (seven subjects) the plaque samples (0.08 - 1.80 mg wet weight) were removed from the same regions and treated in the same manner as the samples for ammonia analysis. Because the urea levels found in this group were quite low, the analyses were repeated on plaques pooled (1.4 - 7.3 mg wet weight) from the same areas of two to five subjects. The latter experiments were performed seven times.

The plaque for either urea or ammonia analysis was dispersed in the HCl by vibration on a Vortex mixer (Scientific Industries, Inc., New York). After centrifugation (11,000xg for two minutes at 25^{o}C), supernatant (20 μl) was transferred to a glass test tube (5 x 60 mm), the

remaining supernatant was carefully removed by suction and discarded. Extraction of the pellet with HCl (25 µl) and the removal of an aliquot of the supernatant (20 µl) was repeated twice. The three 20 µl aliquots were then combined and analyzed for ammonia or urea (see below).

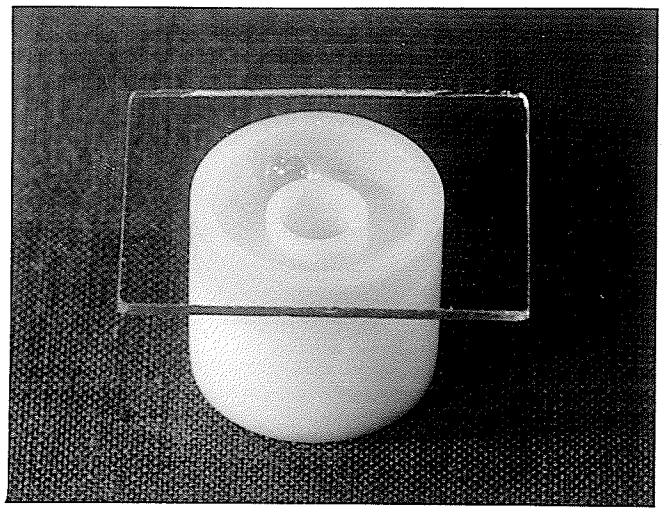
The plaque pellet was digested overnight with H_2SO_4, clarified by adding H_2O_2 and the nitrogen determined as ammonia by Nesslerization (HAWK et al., 1954).

Analytical procedures

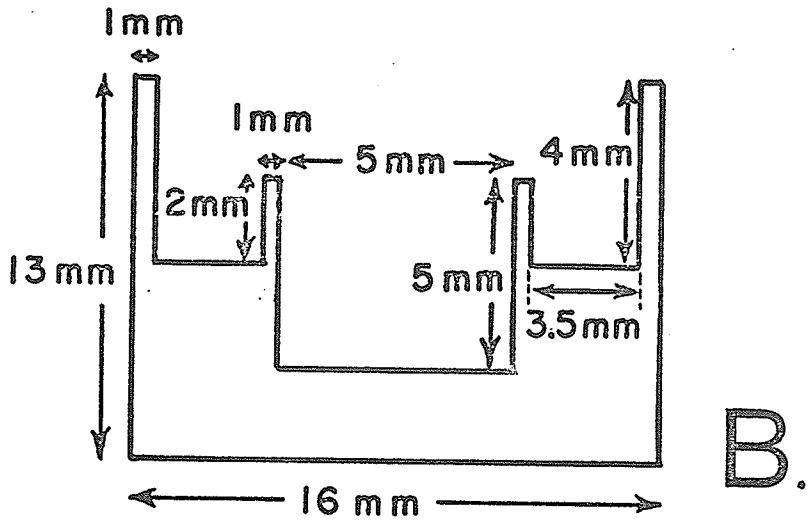
(a) Ammonia

Ammonia was analyzed by an ultramicro adaptation of the micro-diffusion technique of CONWAY (1962). Teflon diffusion dishes were fabricated having the dimensions shown in Fig. 3.2. Boric acid [25 ul, 0.1 percent (W/V)] containing a mixed indicator (RIEF, 1960) was introduced into the inner well; plaque sample and NaOH (1N, 20 ul) were added to the opposite sides of the outer well. Since water does not wet Teflon, it was necessary to introduce a small glass bead (2.5 mm diameter) into the outer chamber to facilitate complete mixing of the sample with the NaOH (Fig. 3.1). After the dishes had been covered with a glass coverslip and sealed with vaseline, mixing was done and the sealed dishes were allowed to stand for a minimum period of five hours (25°C) to ensure complete ammonia diffusion. The ammonia trapped in the boric acid in the center chamber was then titrated with HCl (0.5 mM) and compared to standards (NH_4Cl) similarly treated.

To estimate the precision of the method, 61 analyses were chosen randomly from all those determined (values fell between 19 and 253 ug). The standard deviation of duplicates from their means was ± 6 ug; the coefficient of variation was 6.4 percent. The standard



A.



B.

Fig. 3.2. A photograph (A) and schematic diagram (B) of the teflon dishes used in the microdiffusion technique for the determination of ammonia. (Note the glass cover slip used to seal the dish and the glass bead in the outer well used to mix the NaOH and sample once the cover slip had been sealed in place).

deviation for 33 pairs of blanks and standards (range 0 - 255 μg) was ±3 μg ; the coefficient of variation was 2.2 per cent.

(b) Urea analysis

Urea was analyzed by the diacetylmonoxime-thiosemicarbazide method of Coulombe and Favreau (1963) as modified by Biswas and Kleinberg (1971).

(c) Nitrogen analysis

Aliquots (25 μl) of the plaque pellet digests were analyzed for nitrogen by Nesslerization (HAWK, et al., 1954). Plaque wet wt was calculated from the N value by the following formula:

$$\text{wet weight in mg} = \mu\text{g N} \times \frac{10 \times 6}{1000} .$$

The factor 10 converts μg N to dry weight (SILVERMAN and KLEINBERG, 1967a); the factor 6 converts dry weight to wet weight since about 5/6 of the plaque is water (JENKINS, 1966).

Determination of titratable base

Plaque samples from maxillary labial and mandibular approximal surfaces of the anterior teeth were each transferred to test tubes (10 x 75 mm) containing ice-cold deionized water (300 μl) and gently homogenized (30 sec) with a Teflon pestle. An aliquot (50 μl) of each suspension was then transferred to a cup-shaped glass pH microelectrode (G2221C, Radiometer, Copenhagen) and titrated as follows. Either 0.1 N NaOH or 0.1 N NH_4OH was added with a stainless steel wire loop and after each addition the pH was measured by inserting a salt bridge into the contents of the cup microelectrode (cf. Chapter II).

After completion of the titration, the contents of the electrode cup was transferred to a test-tube (10 x 75 mm), digested with concentrated H_2SO_4 (100 µl) and then analyzed for nitrogen. When the titrant was NH_4OH the nitrogen that would arise from the added NH_4OH was subtracted from the total nitrogen value.

RESULTS

Plaque ammonia

The total ammonia values (ammonium ions plus free ammonia) for the plaques taken from the labial and approximal surfaces of the maxillary and mandibular incisors of the various subjects studied are shown in Table III.1 and Fig. 3.3. For comparative purposes, the pH of plaques in the same regions (from the study of Kleinberg and Jenkins, 1964) are shown in Fig. 3.3.

Plaque ammonia levels followed a similar pattern to that observed for pH. Maxillary labial plaques showed the lowest ammonia levels, whereas the mandibular interproximal plaques showed the highest values. This occurred in every subject examined. An analysis of variance indicated that the difference between (i) maxillary and mandibular plaques (ii) interproximal and labial plaques and (iii) mandibular interproximal and maxillary labial plaques are significant ($p < 0.001$).

Plaque urea

The combined results of the urea analyses for individual and pooled plaques from the 4 regions studied are shown in Table III.2. In neither case was there any significant difference between the various sites.

Table III.1. Ammonia concentration of plaques on the labial and interproximal surfaces of the maxillary and mandibular incisors.

Plaque Location	Plaque Ammonia Concentration (nmoles/mgm wet wt) Number of Samples	Mean ± S.E.M.	Range
Maxillary			
Labial	9	39.6 ± 4.6	22.4 - 66.2
Interproximal	9	53.8 ± 7.2	26.0 - 88.9
Mandibular			
Labial	9	52.6 ± 6.0	33.8 - 87.7
Interproximal	9	85.9 ± 9.9	41.3 - 122.7

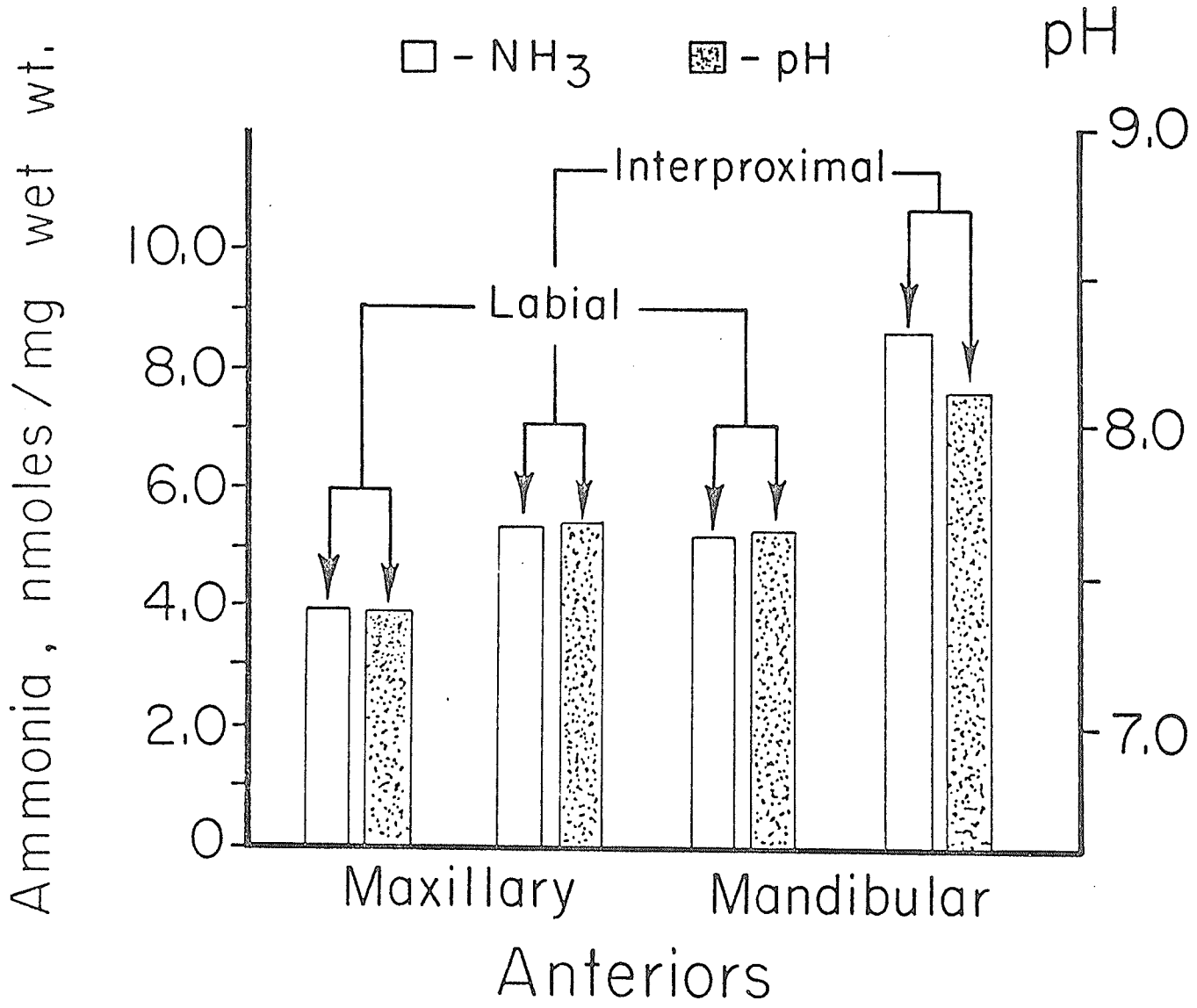


Fig. 3.3. Comparison of the ammonia values and pH levels of plaques on the labial and approximal surfaces of the mandibular and maxillary incisors. (pH values are from the study of Kleinberg and Jenkins, 1964).

Table III.2. Urea concentrations of plaques on the labial and interproximal surfaces of the maxillary and mandibular incisors.

Plaque Location	Plaque Urea Concentration (nmoles/mgm wet wt) Number of Samples	Mean ± S.E.M.	Range
Maxillary			
Labial	14	1.3 ± 0.1	0.5 - 2.5
Interpoximal	14	1.5 ± 0.3	0.6 - 4.1
Mandibular			
Labial	14	1.2 ± 0.1	0.6 - 2.6
Interproximal	14	1.3 ± 0.2	0.6 - 2.3

Titratable base

The titration values found with 0.1 N NaOH and 0.1 N NH_4OH for plaques from maxillary labial and from mandibular approximal sites are shown in Table III.3. The maxillary plaques were titrated between pH 7.10 and 7.39 and mandibular plaques between 7.10 and 8.10. Since 7.39 and 8.10 were the *in situ* pH values found previously for the plaques in these regions of the dentition (KLEINBERG and JENKINS, 1964), the pH range covered in the titrations will consist of 2 parts. The first, between 7.1 and 7.39, is a region of overlap which permitted the buffering capacities of the plaques in the two regions to be compared. The second, between 7.39 and 8.10, is the region of difference. Since the buffering capacities in the pH range 7.1 to 7.39 for the plaques in the two regions were not significantly different ($p > 0.3$, in each case), it was assumed that the buffering capacities between pH 7.39 and 8.1 would be about the same. As a check of this assumption maxillary plaques were titrated between 7.39 and 8.1; the buffering capacity was similar to mandibular plaques over this pH range.

The amount of NaOH needed to raise the pH of mandibular interproximal plaques from 7.39 to 8.1 was 20.8 ± 2.3 nEquiv/mgm wet wt plaque, whereas the amount of NH_4OH needed was 58.3 ± 10.5 nEquiv/mgm wet wt plaque. (These values were obtained by subtracting the values for the buffering capacity of maxillary labial plaques between 7.1 and 7.39 from that of mandibular interproximal plaques between pH 7.1 and 8.1). These values were then compared to the difference in ammonia levels actually measured in these sites (46.3 ± 7.7 nmoles/mgm wet wt plaque;

Table III.3. Comparison of the buffering capacities of maxillary labial and mandibular interproximal plaques.

Plaque Location	pH Range of Titration			
	7.1 - 7.39		7.1 - 8.1	
	with NaOH*	with NH_4OH*	with NaOH*	with NH_4OH*
Maxillary Labial	7.9 ± 1.5	24.2 ± 2.4	24.7 ± 3.4	98.3 ± 12.6
Mandibular Interproximal	9.0 ± 1.6	22.7 ± 4.0	28.9 ± 2.9	82.5 ± 11.2

* nEquivs./mgm wet wt plaque ± S.E.M. (n = 6)

Table III.1). Though these comparisons may suffer from the fact that the titrations, ammonia concentration measurements and plaque pH values were determined on different subjects, it is apparent that the NH_4OH titration value approximates the difference in ammonia levels ($p > 0.3$) better than the titration values with NaOH ($p < 0.01$).

DISCUSSION

This study has shown that ammonia levels are higher in plaques from the mandibular than from the maxillary incisors of fasting subjects. This pattern is similar to that previously found for pH (KLEINBERG and JENKINS, 1964). The largest difference in both cases was between the plaques located on the maxillary labial and mandibular interproximal incisors. Urea levels, on the other hand, showed no such differences.

The similarity between the patterns of plaque pH and plaque ammonia and the difference between the ammonia concentration of mandibular approximal plaques as compared to maxillary labial plaques, supports the hypothesis that plaque ammonia production is responsible for the pH differences (KLEINBERG and JENKINS, 1964). According to this hypothesis, the source of substrate for ammonia formation is salivary urea; however, there is the possibility that urea from crevicular fluid is also involved (GOLUB et al., 1971). The availability of salivary urea will depend upon its concentration in the saliva and the salivary flow rate (KLEINBERG, 1970b). Since mandibular plaques are exposed to greater amounts of saliva than maxillary plaques, the mandibular sites would be exposed to more urea. But the mandibular

anterior sites contain a flora with greater ureolytic activity (ONISI et al., 1957). Consequently, the greater amount of urea available to the mandibular sites would be degraded faster. This could result in higher ammonia and plaque pH levels but urea levels no different than those in plaques where both urea availability and plaque ureolytic activity are less.

The higher incidence of ureolytic organisms in the mandibular plaques is probably the result of the greater urea availability to this region of the dentition.

Significance of plaque ammonia formation from urea

The continuous availability of salivary urea to the plaque bacteria and its degradation would result in the continuous presence of ammonia in the plaque. Since ammonia is toxic to mammalian cells (WARREN, 1962) and since duration of exposure is as important as concentration in determining the intensity of ammonia intoxication (RIZZO, 1967; PRIOR and VISEK, 1972), one might expect that the continuous formation of ammonia in the plaque would have deleterious effects on the gingivae.

Plaque ammonia may also influence bacterial growth. Examination of the inner layers of mature (7 to 13 day) plaque has revealed a high number of dividing coccus-like microorganisms (SCHROEDER and DE BOEVER, 1969). While the lack of certain amino acids has been proposed as a growth limitating factor for bacteria in the depths of plaque (CRITCHLEY, 1969), the anaerobic growth of certain strains of oral streptococci has been shown to occur with ammonia as the major nitrogen

source (CARLSSON, 1970, 1971a and 1972).

Although initially the magnitude of such effects would depend on salivary urea availability, the intensity of these changes may be increased by other processes. For example, the pH may become sufficiently alkaline that the breakdown of proteins and amino acids may be favoured; this in turn would enhance the growth of organisms with ureolytic activity (ONISI et al., 1957). The sum of such effects would be increased plaque ammonia levels.

In the present study, the levels of ammonia found in 3-day plaque (particularly mandibular plaques) are sufficiently high to induce increased flow rates of gingival crevice fluid from clinically healthy gingival crevices (HAYES and HYATT, 1972). This may come about through the plaque ammonia altering the permeability of the sulcular epithelium and stimulating increased flow of crevicular fluid (HAYES and HYATT, 1972). If so, (i) permeation through the crevicular epithelial barrier of those toxic substances which have been implicated in the initiation of gingival inflammation would be facilitated (SOCRANSKY, 1970) and (ii) growth of certain plaque microorganisms would be enhanced by an increased availability of nutrients contained in the crevicular fluid (cf. SALKIND, et al., 1971).

Since crevicular fluid itself contains urea, one would expect that the crevicular bacteria would become more dependent on urea from this fluid and less dependent on urea from saliva as inflammation increased and pocket formation progressed (GOLUB et al., 1971). Nonetheless, it is conceivable that the origin of these processes may

in part be traced back to the situation whereby higher levels of salivary urea availability provided higher levels of plaque ammonia. The rapidity and intensity of such processes may be increased in such disease states as uremia in which the salivary urea levels are markedly increased (HENCH and ALDRICH, 1923; SCHMITZ, 1922) and a generalized stomatitis may occur (CARLIN and SELDIN, 1969). On the other hand, in the healthy individual, these processes would be slower and more obvious in localized regions (ie. those regions where salivary urea availability is higher) and may account for the inverse intra-oral incidence of caries and periodontal disease which has been related to the intra-oral plaque pH and saliva availability patterns (KLEINBERG and JENKINS, 1964).

C H A P T E R I V

UREA, GLUCOSE AND AMMONIA CLEARANCE FROM DENTAL PLAQUE *IN SITU*

Following a glucose rinse, the pH of dental plaque *in situ* rapidly falls. KLEINBERG (1961) attributed this to the rate constant for plaque acid formation being greater than that for acid removal from the plaque. For steady state conditions, STRALFORS (1950) proposed the Acid Production Diffusion (APD) theory. According to this theory, the maintenance of the plaque pH below that of saliva is due to the rate of glucose diffusion into the plaque and its subsequent conversion to acid within the plaque being faster than the rate of acid diffusion out of the plaque into the saliva.

When the plaque is exposed to urea, the pH of the plaque rapidly rises (KLEINBERG, 1967 b). KLEINBERG (1967 b) explained this phenomenon in a similar manner as for glucose; namely, that base formation from urea occurs at a faster rate than base removal from the plaque.

Little quantitative information of these events in dental plaque *in situ* is available, since *in situ* studies on plaque are technically difficult to carry out. MOORE *et al.*, (1956) were able to analyze plaque for lactic acid at intervals following a sucrose rinse and showed that the changes in lactic acid which occurred were coincident with the variations in plaque pH. More recently, GEDDES (1972) found that after consumption of a lump of sugar the plaque concentrations of lactic and volatile acids (probably acetic, propionic and perhaps formic; SANDHAM and KLEINBERG, 1970 b) first increased and then decreased, the volatile acid changes being slower than the changes in lactic acid.

Since more than one acidic substance is formed during glucose degradation (SANDHAM and KLEINBERG, 1970 b) and their proportions are variable and since ammonia is the only basic substance formed from urea, it was felt in the present study that an examination of the acid/base production diffusion properties of the plaque leading to its large changes in pH would be easier to study with urea than glucose. In support of this approach, the experiments in CHAPTER II have indicated that ammonia formation is responsible for most of the base formed and for the pH rise seen in plaque in situ with urea.

The clearance of urea and ammonia from plaque was followed by sampling the plaque before and at intervals after rinsing with a urea solution. For comparison to clearance from plaque, the clearance of urea from saliva was also examined. For comparison of urea to glucose, clearance of glucose from both plaque and saliva were measured.

METHODS

Three-day plaque from fasting subjects was collected as described in the previous chapter. Subjects were instructed not to brush their teeth for three days and to avoid food or drink for at least 12 hours prior to plaque collection which occurred on the morning of the fourth day.

The rinse solution employed was either 0.28 M urea or 0.28 M glucose (BAKER analyzed) warmed to 37°C. After initial samples were taken, each subject rinsed his mouth with two 50 ml portions of rinse solution. The first rinse was held and gently moved about the mouth for one minute and then expectorated. This procedure was immediately repeated with the second 50 ml portion. Plaque samples were then removed with a stainless steel spatula from the labial surfaces of the maxillary lateral and central

incisors at 1, 3, 5, 10, 15, 20, 25, and 30 minutes thereafter.

When the rinse solution contained urea, each plaque sample (about 0.7 mgm wet wt) was dispersed immediately in 50 µl of 0.1 N HCl. After extracting each sample three times with 0.1 N HCL and combining the extracts, aliquots (in duplicate) were analyzed for urea (40 µl) or ammonia (15 µl) by the methods described in CHAPTER II.

When the rinse solution contained glucose, sampling at the 25 minute interval was omitted and each plaque sample (circa 0.5 mgm wet wt) was dispersed in and extracted three times with 25 µl of one percent NaF. An equal volume of 2 percent $ZnSO_4$ and 0.12 N NaOH was then added to 20 µl aliquots (in duplicate) of the combined extracts of each sample. The mixtures were mixed by vibration (Vortex Jr. Mixer, Scientific Industries Inc., New York) and allowed to stand at room temperature for 15 minutes. After centrifugation (1740xg, 15 minutes), aliquots (20 µl) of the supernatant were then analyzed (in duplicate) for glucose using glucose oxidase (KINGSLEY and GETCHELL, 1960). In preliminary experiments testing for the recovery of added glucose to plaque or saliva, the precipitation step involving $ZnSO_4$ and NaOH was found necessary to achieve full recoveries (101.4 $\pm$ 1.6 percent).

The plaque pellet was digested in concentrated H_2SO_4 overnight, clarified by the addition of a few drops of 30 percent H_2O_2 and nitrogen determined as ammonia after Nesslerization (HAWK et al., 1954).

Saliva samples were collected at 1 1/2, 4, 7, 12, 22, and 32 minutes following the rinse by gentle expectoration into glass test-tubes chilled in ice. After centrifugation (1740g for 30 minutes at $4^{\circ}C$), aliquots (10 µl) of the supernatants were analyzed for urea or glucose. Preliminary experiments established that no urea or glucose degradation occurs during the preparation of the saliva samples for analysis.

The experiments were restricted to two subjects, since the difficulty of carrying out these studies necessitated a decision to either do a larger number of experiments on a few subjects or a few experiments on a large number of subjects. One of the subjects (#1) was used to examine plaque and salivary urea clearances following rinsing with urea. The other subject (#2) was used to follow plaque urea and ammonia clearances under exactly the same conditions. Then clearance of plaque glucose in both, and salivary glucose in one of the subjects (#1) was determined after a similar rinse with glucose. The experimental design was such that there was overlap of the variables measured in the two subjects in order to permit some intersubject and intersubstrate comparisons to be made.

RESULTS

(a) Effect of urea rinse on plaque and salivary urea concentrations

Plaque and salivary urea concentrations following a two minute rinse with 0.28 M urea are shown in Fig. 4.1. Both plaque and salivary urea concentrations showed an immediate sharp rise and then a gradual fall, requiring about 20 minutes to reach their base line levels. In all cases, plaque reached its base line level sooner. The logarithms of the urea clearance values for plaque followed a straight line (Fig. 4.2), indicating that disappearance of urea from the plaque is a first-order process. Least-square fitting of the data to the equation of a straight line showed a correlation coefficient, r, of -0.99 ($p < 0.001$).

On the other hand, the logarithm of the urea clearance from saliva did not follow a straight line. During the early part of the experimental period, salivary urea disappeared more rapidly than plaque urea, but subsequently it disappeared more slowly. Measurement of the

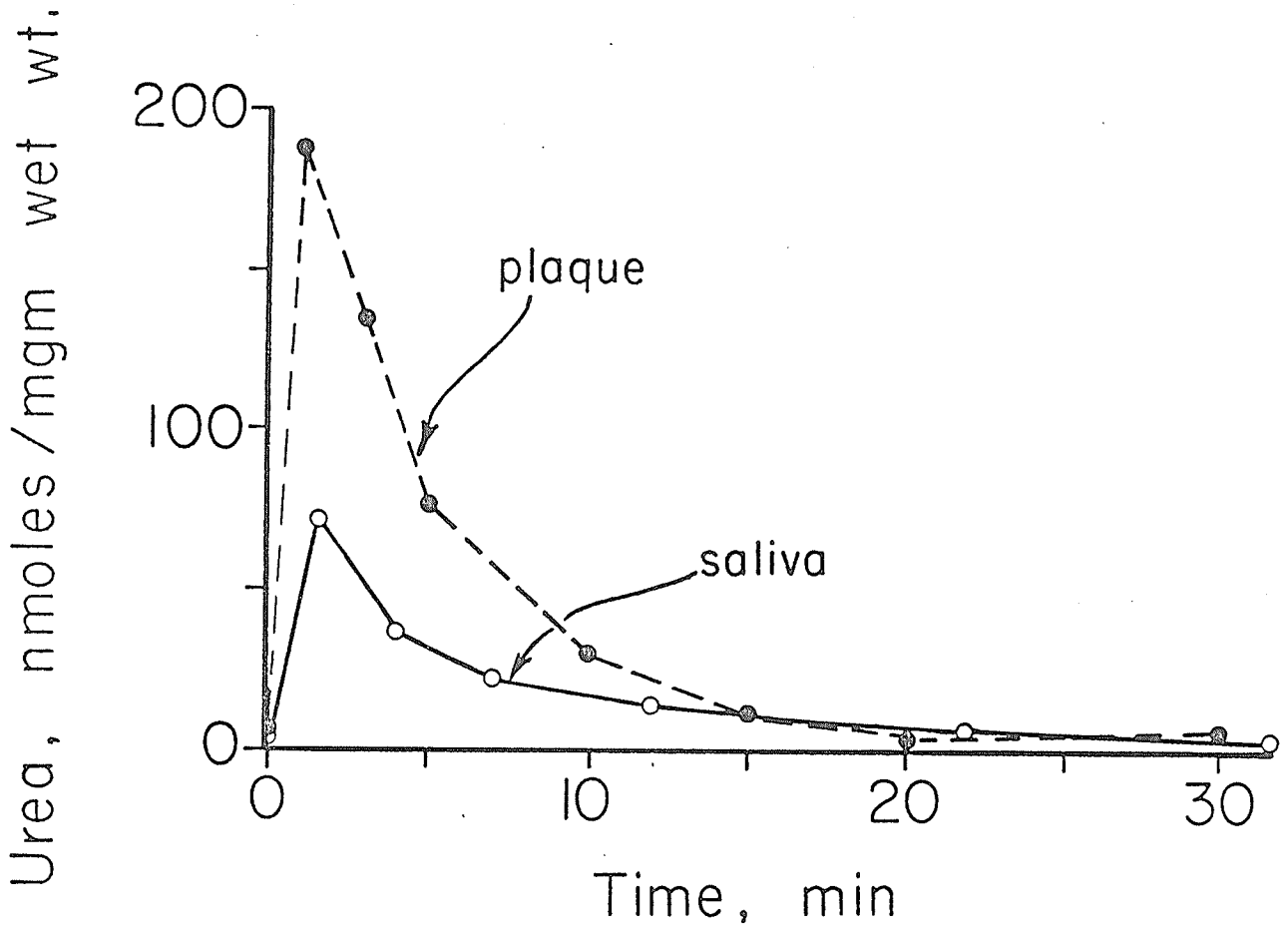


Fig. 4.1. Plaque and salivary urea concentrations before and after a 2 min rinse with 0.28M urea (Subject #1). Each value is the mean of 7 experiments. For plaque values the S.E.M.* = $\pm$ 9 nmoles/mgm wet wt. For saliva values the S.E.M.* = $\pm$ 2 nmoles/mgm wet wt.

*Based on the pooled standard deviation.

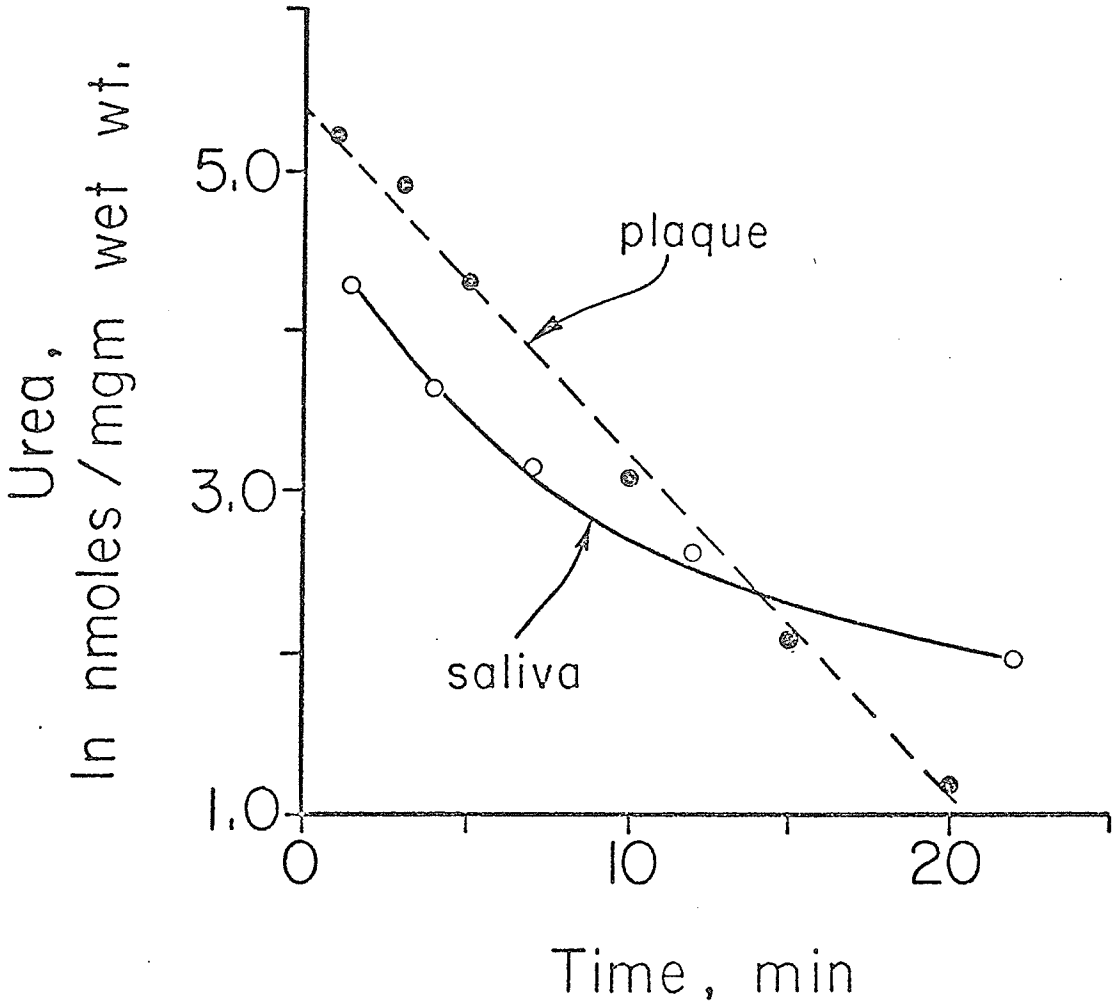


Fig. 4.2. Relation between the logarithms of the urea clearance values and time for both plaque and saliva following a 2 min rinse with 0.28 M urea. The rate constant (k) for the urea clearance from plaque is 0.22 $\pm$ 0.02 (mean $\pm$ S.E.M.) nmoles urea/mgm wet wt/min. The clearance of urea from saliva occurs initially more rapidly than subsequently.

salivary flow rate following the urea rinse showed that an initial high rate which corresponded to the period of rapid salivary urea clearance; subsequently it fell to a steady and slower rate (Fig. 4.3).

(b) Plaque urea and ammonia levels

Plaque ammonia and urea values following rinsing with urea are shown in Fig. 4.4. As in Fig. 4.1, the plaque urea concentration showed an immediate sharp rise followed by a gradual fall to base line levels which occurred within approximately 20 minutes. On the other hand, the plaque ammonia concentration rose to a maximum in ten minutes and then fell; it did not reach base-line values even by the end of the 30 minute experimental period.

The logarithmic values of both the plaque urea concentration after and the plaque ammonia concentration before and after the urea and ammonia reached their respective maxima were plotted against the time as shown in Fig. 4.5. The values for each were fitted to a straight line by the least squares method and the correlation coefficients were as follows: for urea, $r = -0.98$ ($p < 0.001$); for ammonia before its maximum, $r = -0.71$ ($p < 0.05$) and for ammonia after the maximum, $r = -0.85$ ($p < 0.01$). Though no significant difference could be shown between the rate constants for plaque urea clearance and the period of increased plaque ammonia, the difference between the rate constant for ammonia accumulation and the rate constant for ammonia disappearance was highly significant ($p < 0.01$). The difference between urea disappearance and the disappearance of ammonia was also highly significant ($p < 0.01$).

(c) Plaque and salivary glucose levels

For each subject, highest plaque glucose concentrations were

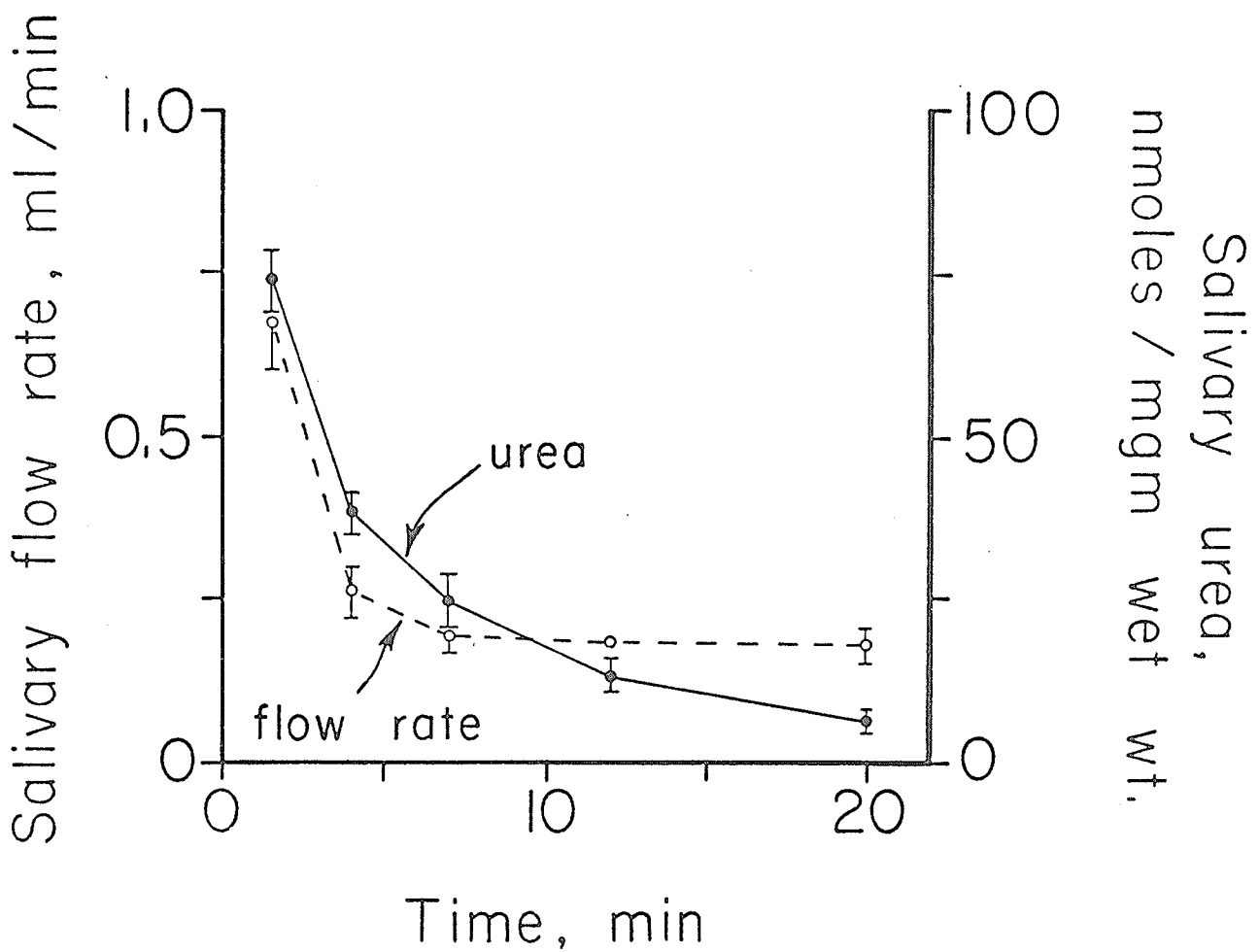


Fig. 4.3. Comparison between salivary flow rate and salivary urea concentration following a 2 min rinse with 0.28 M urea.

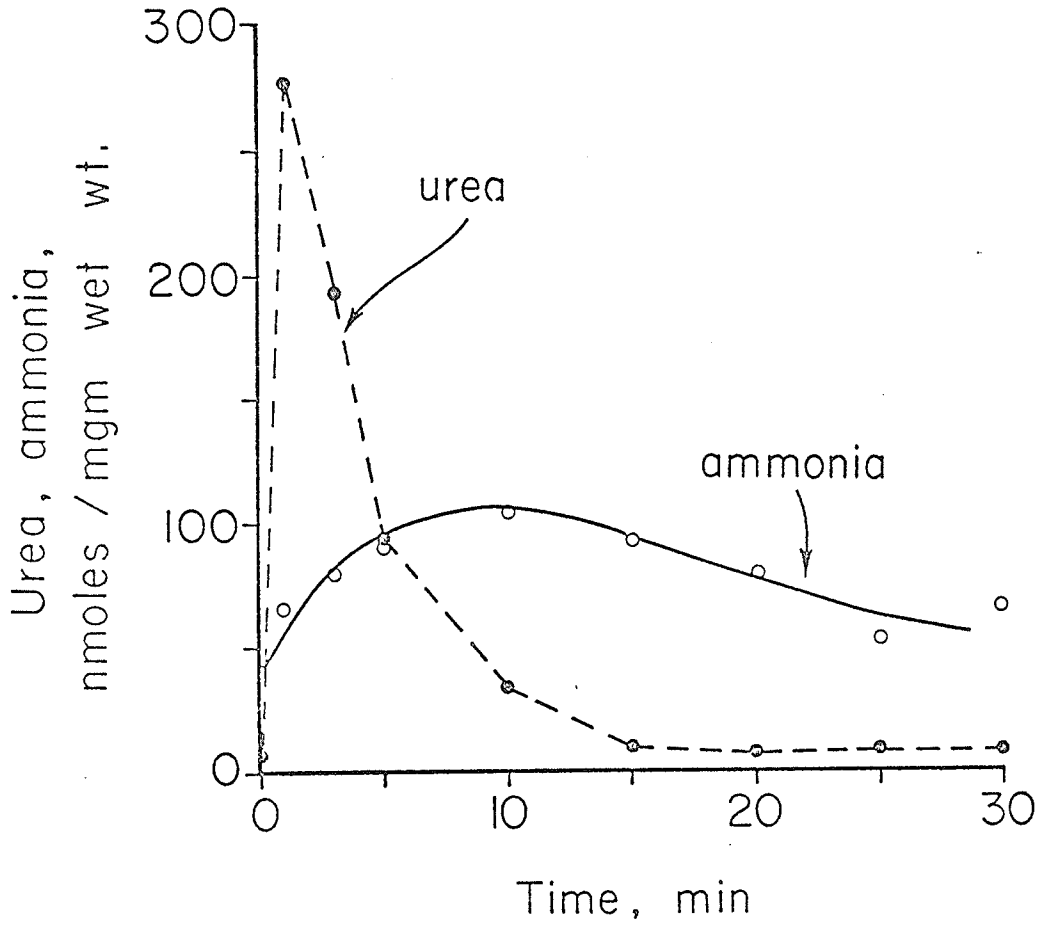


Fig. 4.4. Plaque urea and ammonia concentrations following a 2 min rinse with 0.28 M urea (Subject #2). Each value is the mean of 7 experiments. For the urea values the S.E.M.* = ± 16 nmoles/mgm wet wt. For the ammonia values the S.E.M.*= ± 8.4 nmoles/mgm wet wt.

*based on the pooled standard deviation

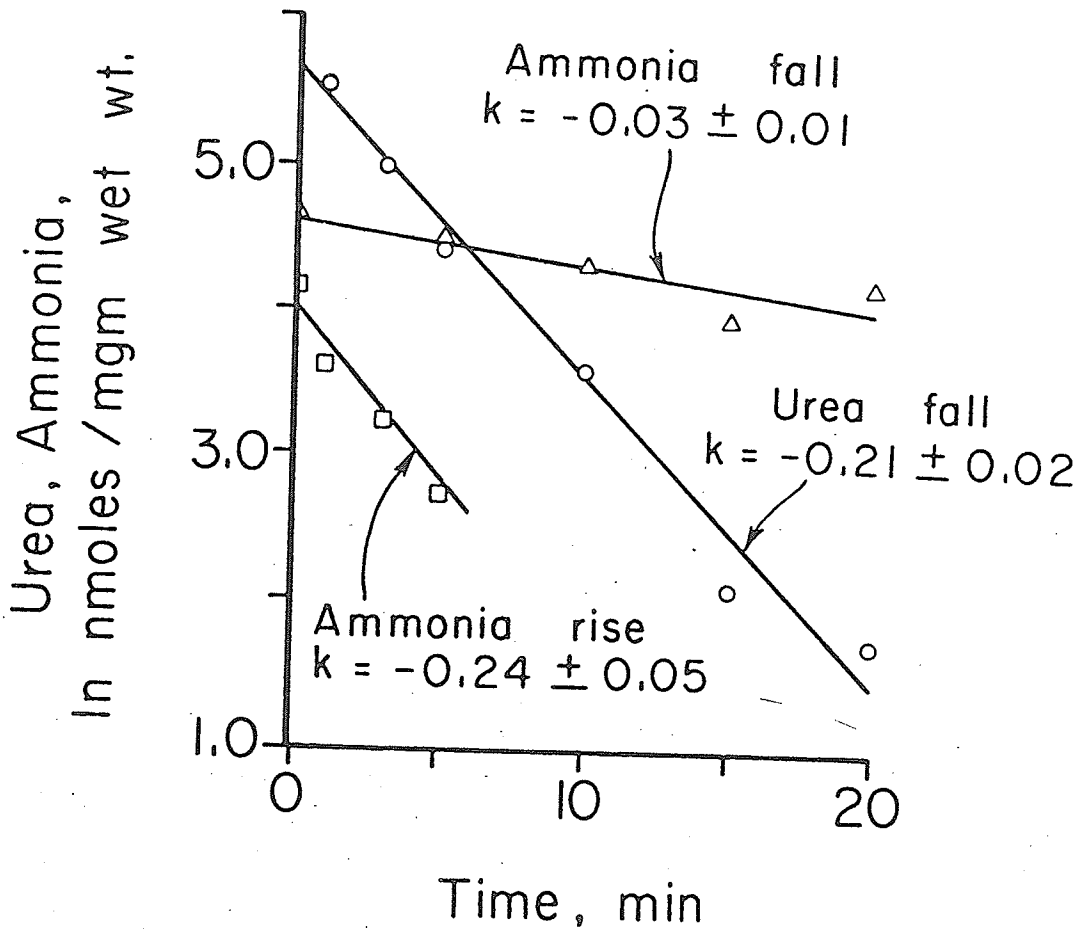


Fig. 4.5. Relation between the logarithms of the urea clearance, ammonia formation and ammonia clearance values, and time in plaque following a 2 min rinse with 0.28 M urea.

(k = rate constant ± S.E.M., nmoles/mgm wet wt/min).

found immediately following rinsing with this substrate (Fig. 4.6); thereafter, the concentration gradually fell and base line values were reached within about 20 minutes. Salivary glucose levels did not reach as high a concentration as plaque glucose levels but reached base line levels between 12 and 22 minutes.

The logarithmic values of the plaque glucose concentrations after the maximum were plotted against the time and compared to the same plot for plaque urea reproduced from Figs. 4.2 and 4.5 (Fig. 4.7). The rate constants for plaque glucose clearance in each of the subjects were similar as was the case for plaque urea clearance. In each subject, the rate constant for plaque glucose clearance appeared to be less than that for plaque urea clearance ($p < 0.1$ and $p < 0.01$, respectively).

The logarithmic values of the salivary glucose concentrations for the period after rinsing were plotted against the time and compared to the same plot of the salivary urea values in Fig. 4.8. Whereas, the clearance of glucose from the saliva was a first-order process ($r = -0.99$, $p < 0.001$), the clearance of urea from saliva was not.

DISCUSSION

The observation in the present study that the rate constant for ammonia formation is approximately eight times greater than the rate constant for ammonia clearance from the plaque would explain why the pH rises so rapidly following plaque exposure to urea. Since no significant difference was found between the rate constants for ammonia formation and the plaque urea disappearance (which would not have been the case if significant amounts of ammonia were lost during this period), these plaque changes probably reflect the conversion of urea to ammonia by the plaque bacteria.

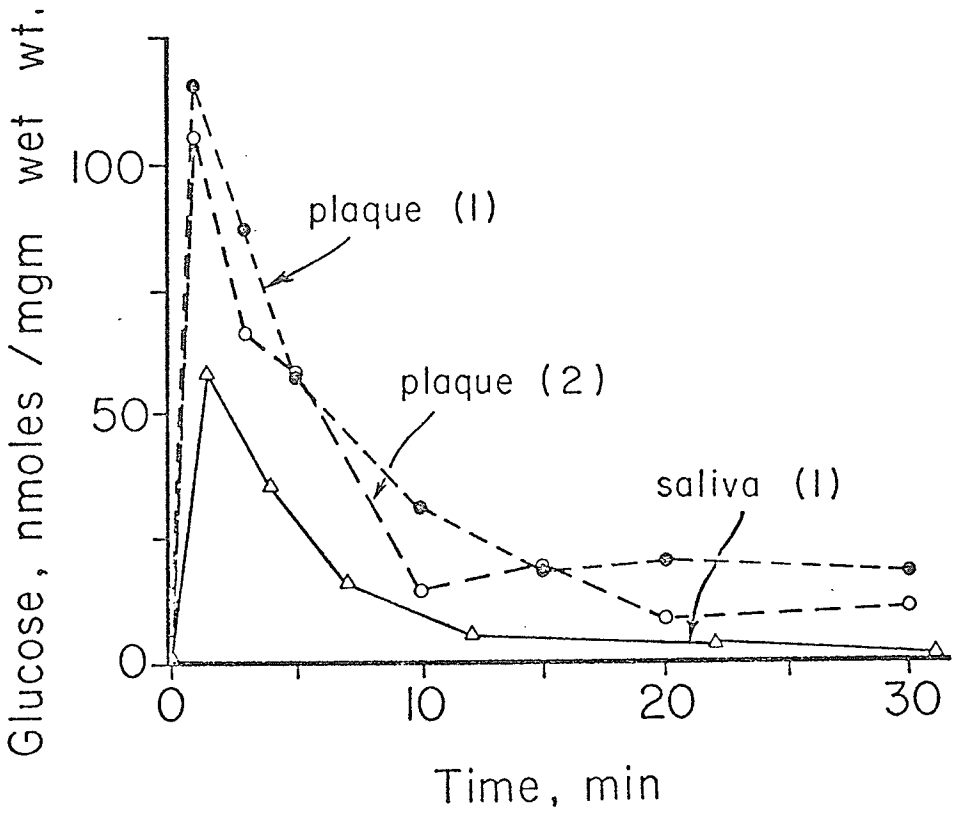


Fig. 4.6. Comparison between plaque (Subjects #1 and #2) and salivary (Subject #1) glucose values after a 2 min rinse with 0.28 M glucose. Each value is the mean of 5 experiments. For the plaque values, the S.E.M.* = $\pm$ 8.9 (Subject #1) and $\pm$ 4.9 (Subject #2) nmoles/mgm wet wt. For the saliva values the S.E.M.* = $\pm$ 5.6 nmoles/mgm wet (Subject #1).

*based on the pooled standard deviation

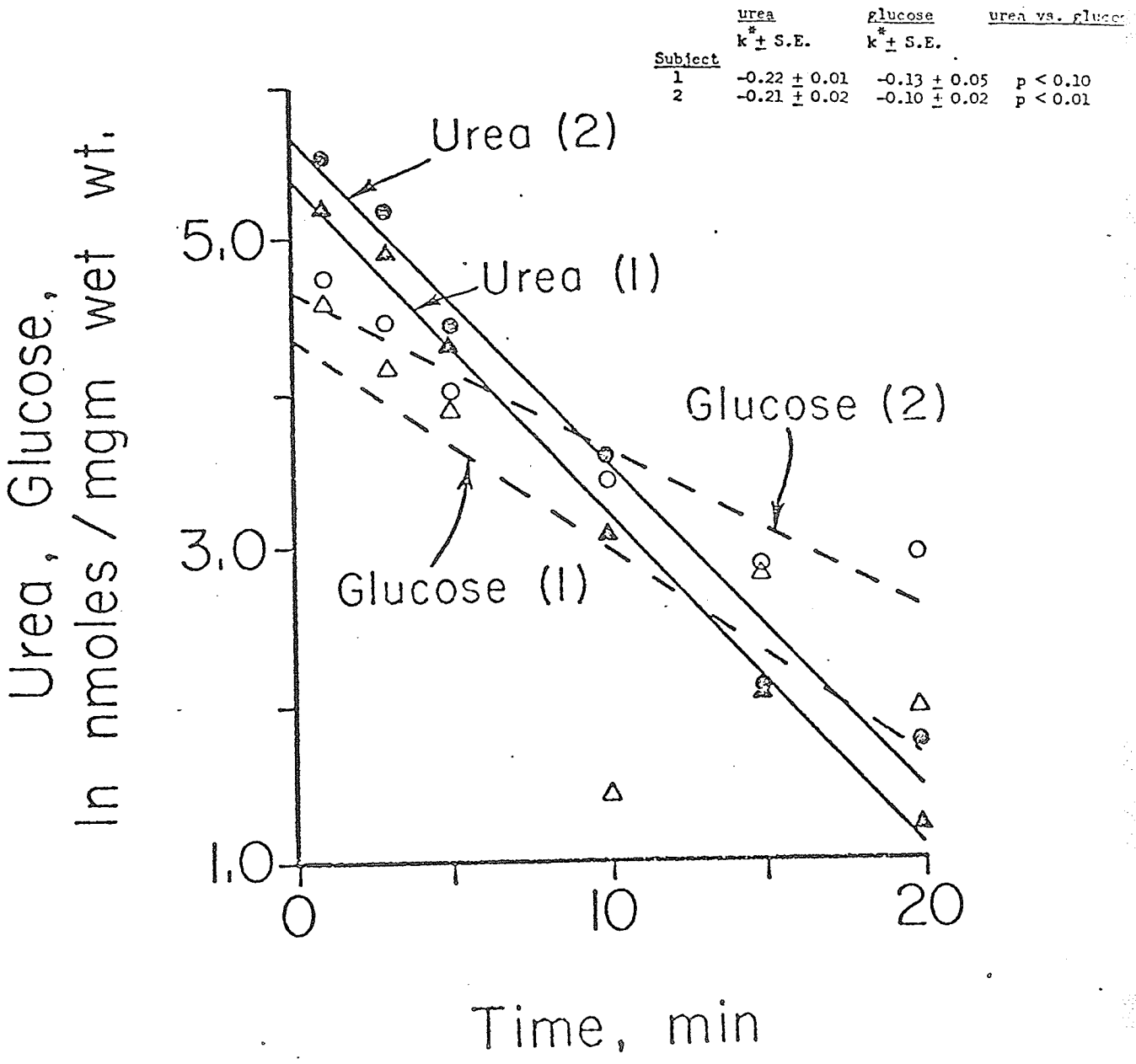


Fig. 4.7. Comparison between the logarithmic clearance values of glucose and urea from plaque following a 2 min rinse with 0.28 M glucose or urea (Subjects #1 and #2). Inset table compares the rate constant for the clearance of urea and glucose from the plaque in each subject; k*=rate constant, nmoles/mgm wet wt/min.

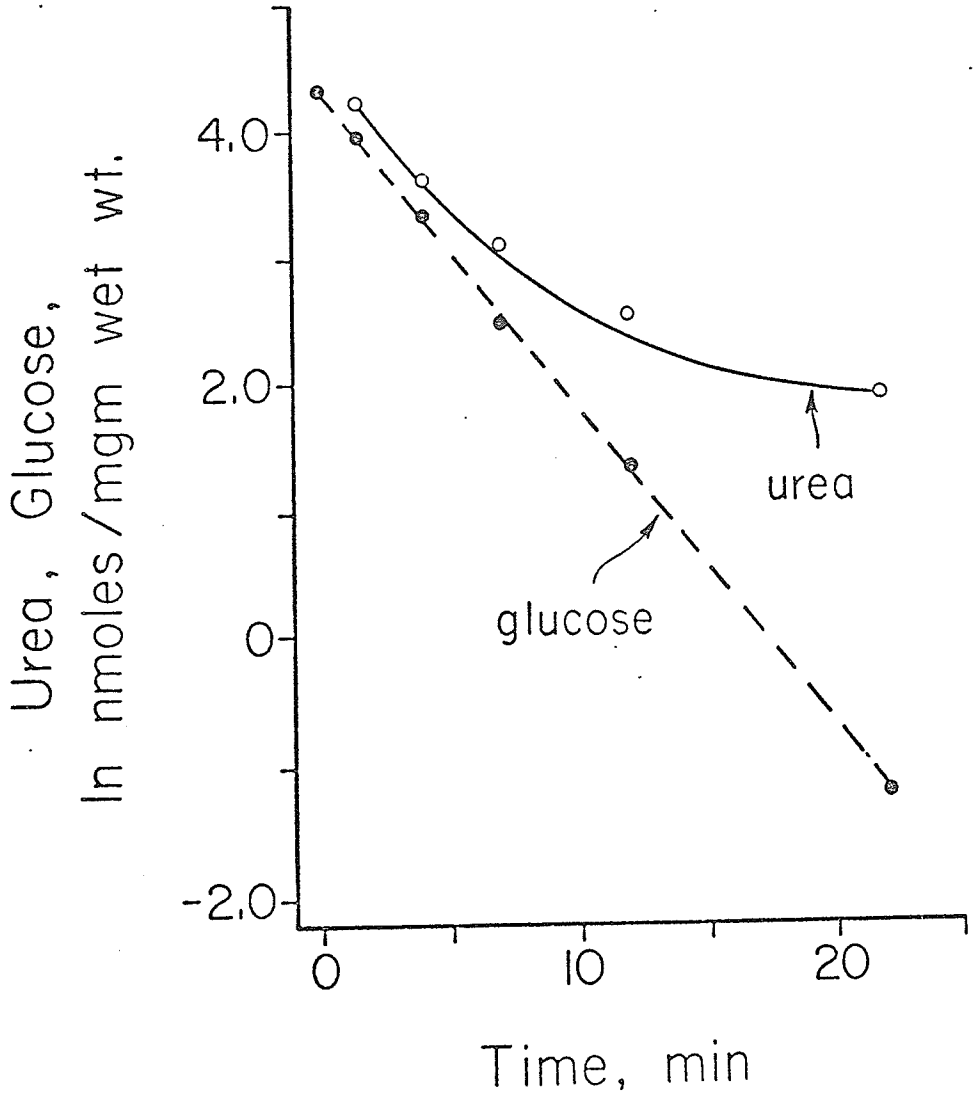


Fig. 4.8. Comparison between the logarithmic clearance values of glucose and urea from saliva following a 2 min rinse with 0.28M glucose or urea (Subject #1).

The ammonia that could have been formed was estimated by substituting the rate constant for plaque urea and ammonia clearance and the urea concentration of the plaque just after the rinse into the equation describing the following series of first-order reactions:

$$Up \xrightarrow{k_1} Ap \xrightarrow{k_2}$$

where Up is the plaque urea concentration, Ap is the plaque ammonia concentration and k_1 and k_2 are the rate constants for plaque urea and ammonia clearance, respectively. The rate expression for this system is:

$$\frac{dAp}{dt} = k_1 Up - k_2 Ap$$

and the integrated form is:

$$Ap = \frac{k_1 Up}{k_2 - k_1} \left[e^{-k_1 t} - e^{-k_2 t} \right]$$

The rate expression is a simplified form of one proposed earlier for explaining the events leading to the pH changes in plaque during glucose or urea degradation (KLEINBERG, 1961 and 1967 b). Allowing for the fact that two moles of ammonia theoretically can be formed from one mole of urea, the amount of ammonia actually found was 16 - 26 percent of the value calculated for Ap (Table IV.1). This suggests that as in salivary sediment, the remainder is stored in the form of amino acids synthesized de novo or incorporated into protein (KLEINBERG, 1967 b; BISWAS and KLEINBERG, 1971).

Uptake of urea and glucose by plaque.

The higher concentrations of urea and glucose reached in the plaque than in the saliva immediately following rinsing indicates that uptake of these substrates by the plaque is a rapid process. This would not be the case if the plaque was gel-like in structure where diffusion

Table IV. 1. Comparison of the ammonia measured in plaque following the urea rinse to that which could have been formed during this time period.

Time (Min)	Ammonia Formation (nmoles/mgm wet wt) Theoretical	Measured*	Measured as per cent of theoretical
1	104.4	27.2	26
3	248.1	39.6	16
5	333.0	59.5	18
10	404.8	65.7	16

* measured amount of ammonia = total ammonia in plaque minus pre-rinse baseline value.

is a relatively slow process (STRALFORS, 1950). It would certainly be the case if the basic structure of the plaque was a floc (through which percolation is rapid), as the aggregation experiments of SILVERMAN and KLEINBERG (1967) on dental plaque suggest.

In both subjects, the plaque urea concentration immediately following the rinse reached approximately twice the value reached with glucose, suggesting that the movement of urea into the plaque is faster than the movement of glucose. If so, such a difference might be due to the difference in their size. In support of this possibity is the observation that the diffusion coefficient of urea in water (1.18 [cm^2/sec] x 10^5) is almost twice that of glucose (equal to 0.60 [cm^2/sec] x 10^5; HOBER, 1945). Assuming this relationship also applies with plaque fluid (EDGARS, 1972), these results with urea and glucose would support the contention of STRALFORS (1950) and KLEINBERG (1961 and 1967 b) that diffusion is the rate-limiting step in the uptake of these substrates by the plaque.

Clearance of urea and glucose from plaque

After the rinse, urea or glucose may be cleared from the plaque either by diffusion, bacterial degradation, or a combination of the two processes (KLEINBERG, 1970 a). The fact that urea clearance from the plaque paralleled plaque ammonia accumulation but not the clearance of urea from saliva, indicates that plaque urea clearance is mainly metabolic. Since the plaque samples were restricted to the labial surfaces of the maxillary anterior teeth, an area which has comparatively poor access to saliva (STEPHAN, 1943; KLEINBERG and JENKINS, 1964) and since the subjects minimized their oro-facial movements during the experiments, the contribution of saliva to the clearance of substrate from these

plaques was probably near its lowest limits (LANKE, 1957; NIVEN, 1954).

If the clearance of substrate reflects mainly its utilization by the plaque bacteria, then the faster utilization of urea than glucose is due either to (i) the presence of greater numbers of ureolytic than glycolytic organisms or (ii) the uptake and degradation of urea by the paque bacteria being more rapid than that of glucose.

With regard to the first point, very little information is available. Although the plaque has considerable ureolytic activity, the microorganisms responsible for this activity have not yet been determined. ONISI et al (1957) found variation in the incidence of ureolytic bacteria and as a result variation in the ureolytic activity of plaques from different regions of the human dentition. The incidence ranged from a high of 72.7 percent in plaques taken from the mandibular interproximal incisor regions to 27.2 percent in the maxillary interproximal bicuspid region. While staphylococci seem to be the most active ureolytic organisms in plaque, the amount of staphylococci present and capable of colony formation on Difco Staphylococcus Medium 110 is not sufficient to account for the ureolytic activity of dental plaque (FROSTELL, 1960). Ureolytic activity has been claimed for aerobically grown oral streptococci, neisseriae and diphtheroids (ONISI et al., 1957). However, FROSTELL (1960) could not duplicate these findings. Obviously, the determination of the plaque microorganisms responsible for its ureolytic activity needs re-investigation.

With regard to the difference in uptake of urea and glucose by the plaque, studies in vitro (KLEINBERG, 1961 and 1967b; BISWAS and KLEINBERG, 1971) indicated that plaque utilizes urea more rapidly than glucose and that entry of the urea into the plaque is faster than that

of glucose.

With regard to relative rates of degradation, FROSTELL (1960), using plaque suspensions, compared the relative rates of ammonia production and lactic acid formation from urea and glucose, respectively (FROSTELL and RHODIN-BLOMBERG, 1957). From these investigations he estimated that ammonia is produced about ten times faster than lactic acid. This may be somewhat of an overestimation since the plaque was incubated without saliva and saliva has a marked enhancing effect on glycolysis but only a small effect on urea degradation (KLEINBERG et al., 1973; CHAPTER II). Nonetheless, these findings would suggest that similar differences occur in plaque in situ and may account in part, at least, for the more rapid clearance of urea than glucose from plaque found in the present study.

Clearance of urea and glucose from saliva

The clearance of either urea or glucose from saliva was different from the clearance of each from the plaque.

Urea clearance from saliva was multiphasic, whereas from plaque it was a first order process (Fig. 4.2). Although many factors can influence its clearance from saliva (cf. LANKE, 1957), the results in the present study suggest that the change in salivary flow rate after the urea rinse - rapid initially and slower subsequently - would have a major influence on the dilution rate and therefore clearance of the urea from the saliva (Fig. 4.3).

On the other hand, the clearance of glucose from saliva was a first-order process and faster than its clearance from plaque. This may indicate in the case of glucose that diffusion is more significant than degradation in the clearance of this substance from the plaque.

One should keep in mind that the salivary clearance values might be influenced by retention of glucose or urea in regions of the mouth such as the tongue, cheeks and the gingivae. These values might have also been influenced by consumption of glucose or urea by the masses of bacterial cells found on the attached gingivae (KLEINBERG et al., 1971; HALHOUL and COLVIN, 1973), since measurement of the pH response of these surfaces following exposure to sucrose indicated that the bacteria on these surfaces can readily utilize sucrose and produce acid from it (KLEINBERG et al., 1971).

Clearance of plaque ammonia

The slow clearance of ammonia from the plaque could involve at least two processes. Firstly, at the plaque pH values after the urea rinse (see CHAPTER V) the majority of the ammonia would be in its NH_4^+ form and more likely to interact with the charged groups of the plaque matrix protein. Consequently, the diffusion of the ammonium ion out of the plaque may be impeded. Secondly, ammonia formation may continue even after the urea is completely utilized, since ammonia could come from the de-amination of amino acids (BISWAS and KLEINBERG, 1971; CHAPTER V).

CHAPTER V

THE EFFECT OF GLUCOSE AND UREA ON THE COMPOSITION OF THE AMINO ACID POOL OF DENTAL PLAQUE *IN SITU*

Cells contain a pool of free amino acids that changes in composition and size in response to alterations in the extracellular environment (DAWSON, 1965; TEMPEST *et al.*, 1970). During the growth of pure cultures of bacteria, the size of the intracellular pool does not vary as much as its amino acid composition (TEMPEST *et al.*, 1970). Changes in composition usually occur when the availability of carbon or nitrogen sources are altered. This is the case whether or not the growth conditions are restricted by limiting the availability of substrates such as glucose and ammonia or non-restricted by having them in excess (TEMPEST *et al.*, 1970). On the other hand, in the absence of exogenous substrates, the size of the free amino acid pool (easily released under these conditions) increases greatly, changing much more than the composition (MANDELSTAM, 1958). This last finding has been related to the degradation of cellular protein which occurs more readily under non-growth than under growth conditions (MANDELSTAM, 1958).

Similar alterations have been observed in studies with salivary sediment (BISWAS and KLEINBERG, 1971; CRAW and KLEINBERG, 1971). During incubation with urea, most of the urea-N was stored and the concentration of several of the amino acids in the free amino acid pool increased, indicating that *de novo* synthesis may have taken place. Once the available urea was completely utilized, several of the amino acids which had increased while the urea was available, now decreased. In the presence of glucose, urea stimulated a large increase in the level of alanine (BISWAS and KLEINBERG, 1967).

While examining the influence of glucose and salivary supernatant on the free amino acids of salivary sediment *in vitro* only certain amino acids showed large changes in concentration in the presence of glucose and a favourable pH for glucose utilization (CRAW and KLEINBERG, 1971). However, without glucose or in the presence of an unfavourable pH (below pH 5.0 or above pH 9.0) the concentration of most amino acids progressively increased. CRAW and KLEINBERG (1971) therefore concluded that degradation of some, if not all of the protein is prevented by the availability of glucose.

The change in the pool that occurred in the absence of exogenous substrate in one study (CRITCHLEY, 1969) suggested that plaque proteases degraded proteins of the plaque matrix. Under these circumstances, one would expect that in addition to intracellular amino acids, the plaque pool would also contain amino acids from such protein or protein from cells.

Since very little is known regarding the changes in the plaque pool of free amino acids, the present study has examined: (i) whether the amino acid pools of plaques located in different regions of the dentition differ in their composition (ii) whether exposing plaques *in situ* to glucose, urea or both substances at the same time alters the composition and size of the pool and (iii) whether the amino acid pool differs from that obtained if plaque or components of the plaque were to undergo hydrolysis.

Experiments were carried out in which plaques located on the labial and interproximal surfaces of the maxillary and mandibular incisors were analyzed for free amino acids. These areas were selected because of (i) the large differences in their access to saliva and therefore to

salivary urea and (ii) the indication from earlier studies that mandibular plaques may have a more active urea metabolism than maxillary plaques. Evidence for the latter includes the fact that mandibular plaques show a higher pH (KLEINBERG and JENKINS, 1964), higher levels of ammonia (CHAPTER II) and higher ureolytic activity (ONISI et al., 1957) than maxillary plaques.

Experiments were then carried out in which the effects of urea, glucose or glucose-urea rinses on the composition of the amino acid pool were explored. For reference to earlier studies (KLEINBERG, 1961, 1967b) the changes in plaque pH with these rinse solutions was also determined.

Finally, the amino acid composition of acid hydrolysates of plaque bacteria was investigated. This and the amino acid composition of acid hydrolysates of plaque matrix components (SILVERMAN and KLEINBERG, 1967a) were compared to the plaque pool of free amino acids to determine whether hydrolysis of plaque bacterial or matrix protein contributes significant amounts of amino acids, to the plaque pool.

METHODS

(a) Analysis of the amino acid pools of plaques located on maxillary and mandibular incisors

Subjects (20) were divided into 4 equal groups. Each group of subjects was instructed to stop all oral hygiene procedures for 3 days and avoid eating or drinking for 12 hr prior to the time of plaque collection, which was done on the morning of the fourth day. At time time of plaque collection each subject held his head erect and lips closed until the moment of plaque removal. The lips were then retracted gently to ensure minimal saliva stimulation. The plaque, after removal with a stainless

steel spatula, (circa 5.0 mgs wet wt) was pooled according to location in test-tubes containing 500 µl of cold 0.1 N HCl (HANCOCK, 1958). The plaque material was dispersed immediately by vibrating on a vortex mixer (Scientific Industries Inc. New York). After 15 min, the plaque suspension was centrifuged (1740xg, 15 min), the supernatant decanted and the plaque pellet extracted again with 250 µl of 0.1 N HCl. Analysis of the first and second extracts showed that the amino acids contained in the second extract were only 3 per cent of that present in the first.

The extracts were combined, dried under a stream of filtered air, and then stored at $-10^{o}C$ until analyzed for amino acids (see below).

Concentrated H_2SO_4 (Baker Analyzed) was added to the plaque pellets which were then heated to digest the plaque protein prior to analysis of nitrogen (see below).

Comparison of 0.1 N HCl as the extracting fluid to 5 per cent trichloracetic acid and to 75 per cent ethanol revealed that all of the solutions extracted similar proportions but different total amounts of amino acids; HCl extracted the most.

(b) Effect of rinsing with glucose and/or urea on the amino acid pools

These experiments were restricted to plaque found on the labial surfaces of the maxillary incisors of a single subject (D.L.S.). A total of 15 experiments were carried out. Their design was similar to that used to follow the clearance of urea and glucose from the plaque (Chapter IV).

The rinse solution in each experiment was either 0.28 M urea, 0.28 M glucose, or 0.28 M urea and 0.28 M glucose in 1:1 ratio, or distilled water. Four experiments were performed with each rinse solution, except for urea with only three experiments being performed. A table of random numbers was used to allocate the order of the various rinse experiments

and the sites (ie. the 4 incisors) from which plaque would be collected at the different times in the different experiments.

A sample of plaque was removed before the subject rinsed his mouth with 2 portions (50 ml) of the rinse solution which was previously warmed to 37°C (cf. CHAPTER IV). The subject gently moved each portion about his mouth for one min before expectorating. At intervals of 1, 10 and 20 min, thereafter, plaque (circa 2.8 mgm wet wt) was removed, extracted twice with 0.1 N HCl (100 ml) and the combined extracts were then analyzed for amino acids (see below).

In similar rinse experiments, the plaque pH was measured with an antimony pH micro-electrode as described previously (KLEINBERG, 1958).

(c) Amino acid composition of a hydrolysate of plaque cells

The amino acid composition of hydrolysates of the bacterial component of dental plaque was examined as follows. Using the procedure of SILVERMAN and KLEINBERG (1967a), 3-day plaque from 5 subjects was pooled and suspended in cold 0.1 N NaOH in order to separate most of the bacteria from the matrix material. The suspension was then centrifuged (12,800 for 10 min at 4°C) and the bacterial pellet after collection was hydrolyzed in 6 N HCl (118°C for 18 hr *in vacuo*). Two aliquots (100 µg N) of hydrolysate were each analyzed for amino acids.

(d) Analytical procedures

(i) amino acid analysis

The samples from experiments (a) and (c) were analyzed on an NC-1 70 cm Technicon amino acid analyzer, the much smaller samples from experiment (b) were analyzed on a TSM-1 amino acid analyzer (Technicon Corp., Tarrytown, New York), which could be run at 10 times the sensitivity of the NC-1 instrument. This method, however, was less equivocal in the

estimation of several amino acids.

(ii) nitrogen analysis

Nitrogen was determined by Nesslerization following digestion of samples with concentrated H_2SO_4 (HAWK et al., 1954). Plaque N values were converted to plaque dry weight by multiplying by 10 (SILVERMAN and KLEINBERG, 1967a) and to plaque wet weight by further multiplying by 6 (JENKINS, 1966; cf. CHAPTER III).

RESULTS

Composition of the free amino acid pools of the incisor plaques studied

The composition of the pool of free amino acids extracted from maxillary labial incisor plaques sampled on 15 separate occasions in a single subject is shown in Fig. 5.1. Glutamic and aspartic acids, ornithine, lysine, proline, alanine, glycine, threonine, and serine were the amino acids found most consistently. On occasion, trace amounts of valine, arginine, isoldeucine, leucine, histidine, tyrosine, methionine and phenylalanine could be detected. Glutamic acid alone constituted at least 50 percent of the total pool.

The amino acid composition of pools from the different incisor sites of four groups of subjects (five persons per group) is shown in Fig. 5.2. The pattern for plaques from each site was similar to that shown in Fig. 5.1. In all cases, both glutamic and aspartic acids were present in largest amounts. However, there was a tendency for both glutamic and aspartic acids to be highest in mandibular interproximal plaques. For these amino acids, the differences between mandibular interproximal and mandibular labial and mandibular and maxillary interproximals were both significant ($p<0.05$); the difference between mandibular interproximal and maxillary labial plaques was not.

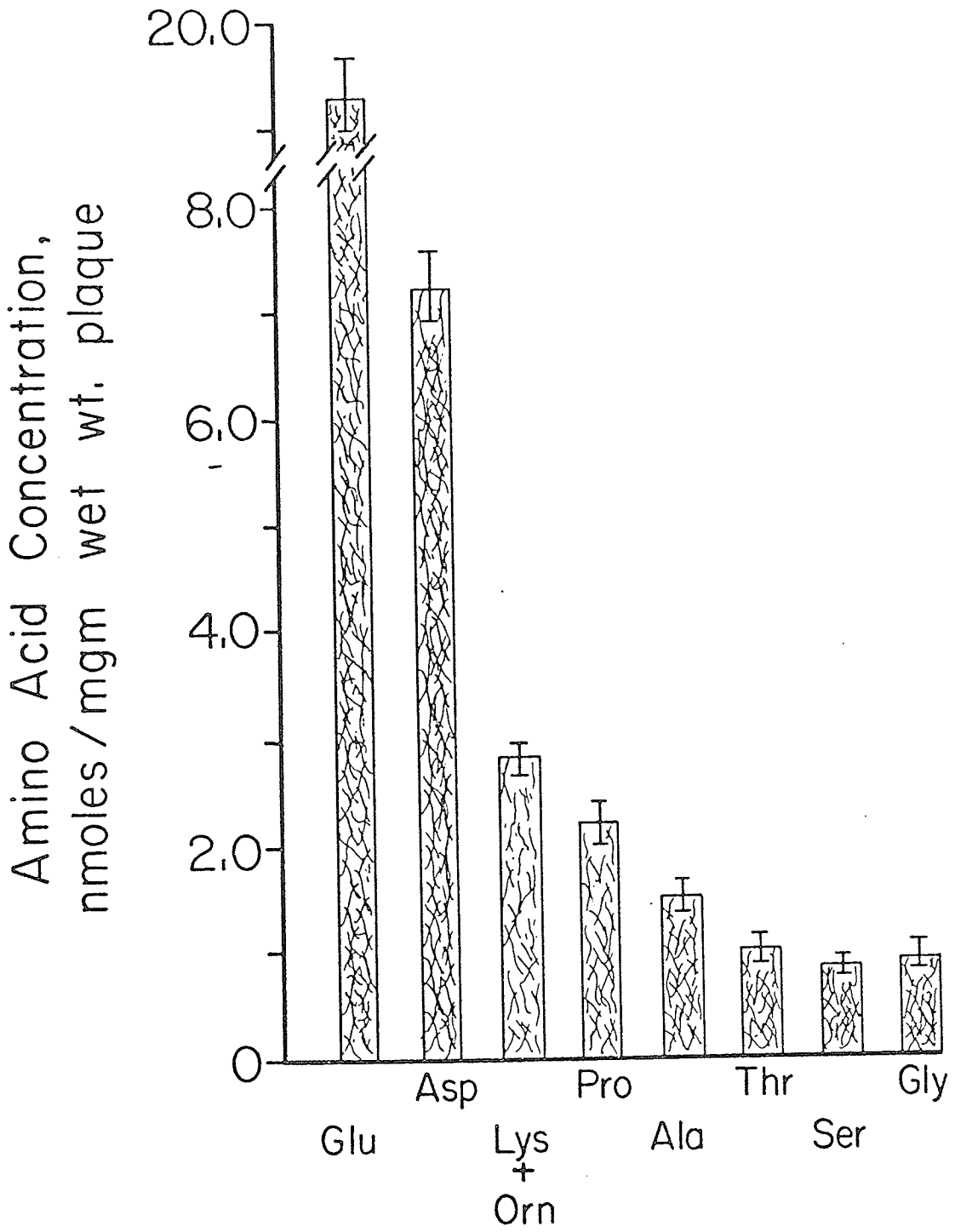


Fig. 5.1. The composition of the pool of free amino acids of plaques located on the labial surfaces of the maxillary incisors. A single subject was used and the values shown are the mean ± S.E.M. of 15 samples, collected on 15 different occasions.

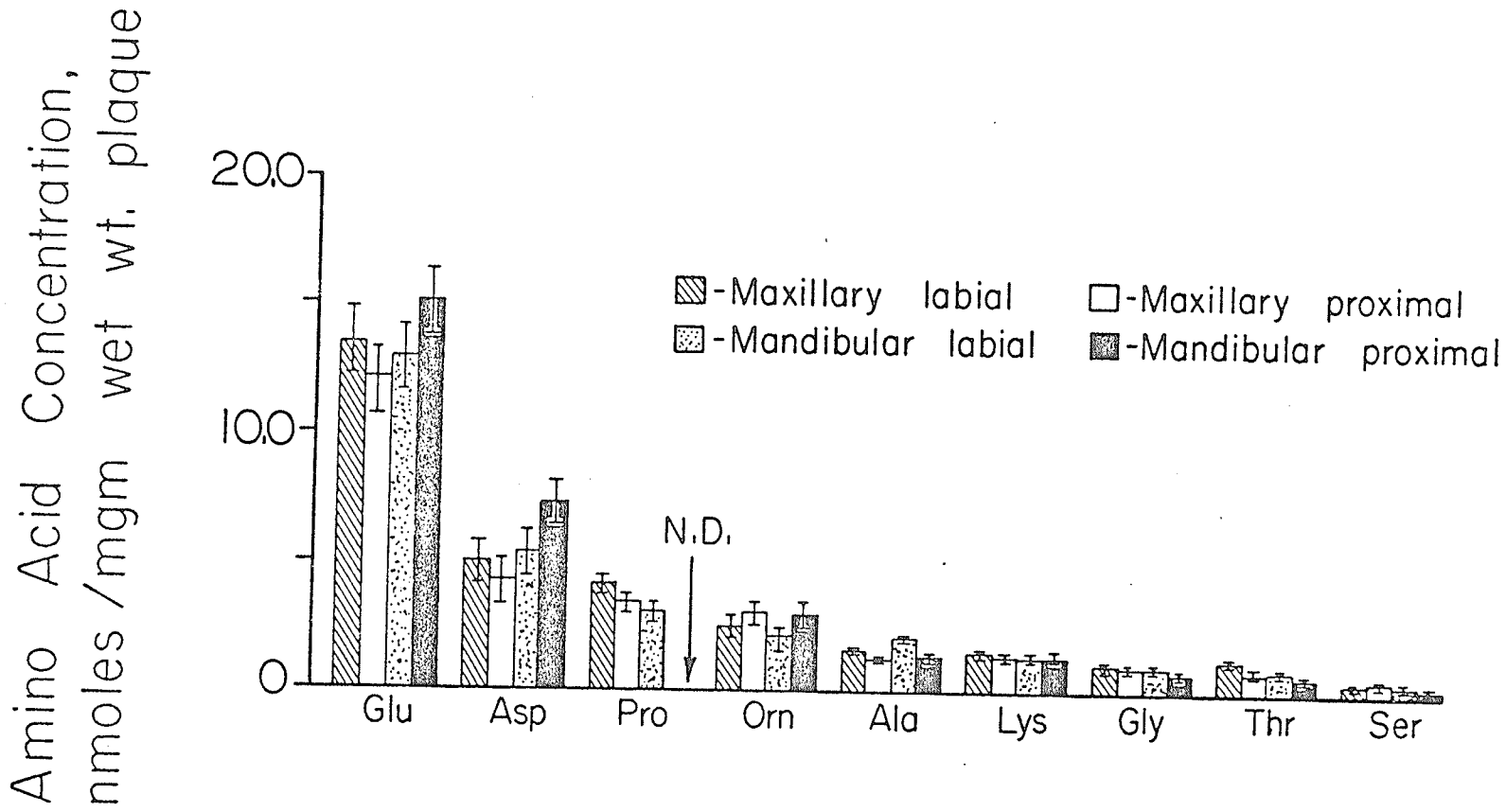


Fig. 5.2. The composition of the free amino acid pools of plaques located on the labial and proximal surfaces of maxillary and mandibular incisors. In these experiments, proline was not detected (N.D.) in plaques from mandibular proximal sites. However, in other experiments using the TSM-1 Technicon Amino Acid Analyzer, proline could be detected at a similar level in all sites.

No proline could be detected in the mandibular interproximal plaques in this series of analyses. One possibility was that the smaller amounts of plaque available from mandibular interproximal areas for sampling (average of 3.6 mg wet wt as compared to 6.71 mg wet wt from the other sites) and the poor sensitivity of proline with ninhydrin may have prevented its detection. When larger amounts of plaque from this and the other sites were collected and pooled from several individuals and the extract analyzed for proline on the TSM-1 Technicon Amino Acid Analyzer; proline could be detected and was at a similar level in all sites.

Effect of urea, glucose, urea plus glucose or distilled water on the composition and size of the free amino acid pool

Following the water rinse, the total pool immediately decreased slightly and then returned gradually to pre-rinse values (Fig. 5.3). Since the proportions of the amino acids in the pools did not markedly change, (Fig. 5.4), this suggests that the water rinse simply washed out a small amount of the pool amino acids. It also suggests that amino acid values following the other rinses would actually be somewhat higher than measured for those that show an increase in level and not quite as low for the amino acids showing a decrease.

Much larger changes in the size of the pool occurred following the substrate rinses (Fig. 5.3). After exposure of the plaque to urea, the pool immediately increased in size and was sustained at an elevated level for at least 20 min. On the other hand, when glucose was the rinse solution, the size of the pool decreased. The rinse solution containing both urea and glucose resulted in the pool increasing during the first 10 min in a manner similar to that observed with the urea rinse; thereafter, the pool decreased and approached pre-rinse values.

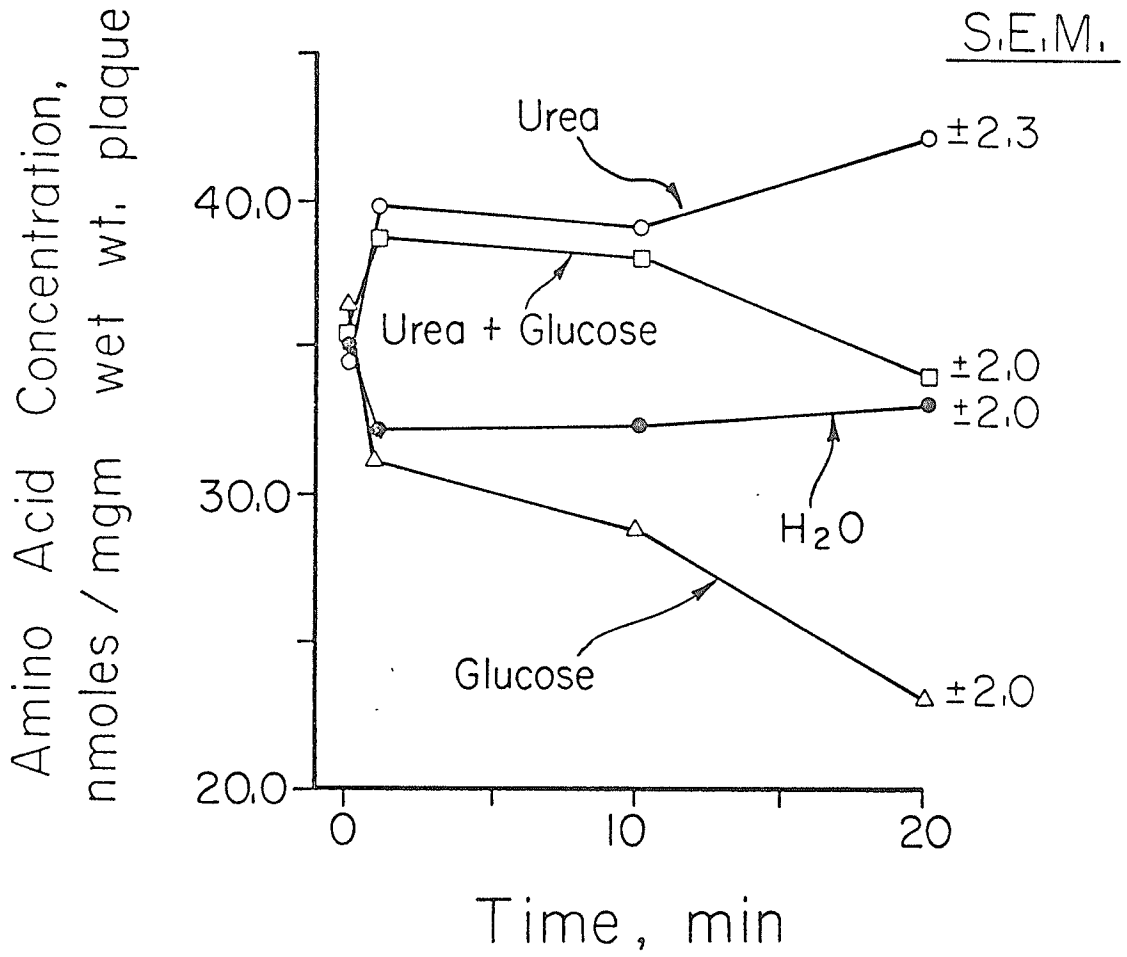


Fig. 5.3. Changes in the size of the total pool of plaque free amino acids following a rinse with either (i) urea (0.28 M), (ii) glucose (0.28 M), (iii) glucose-urea (each 0.28 M) or (iv) distilled water.

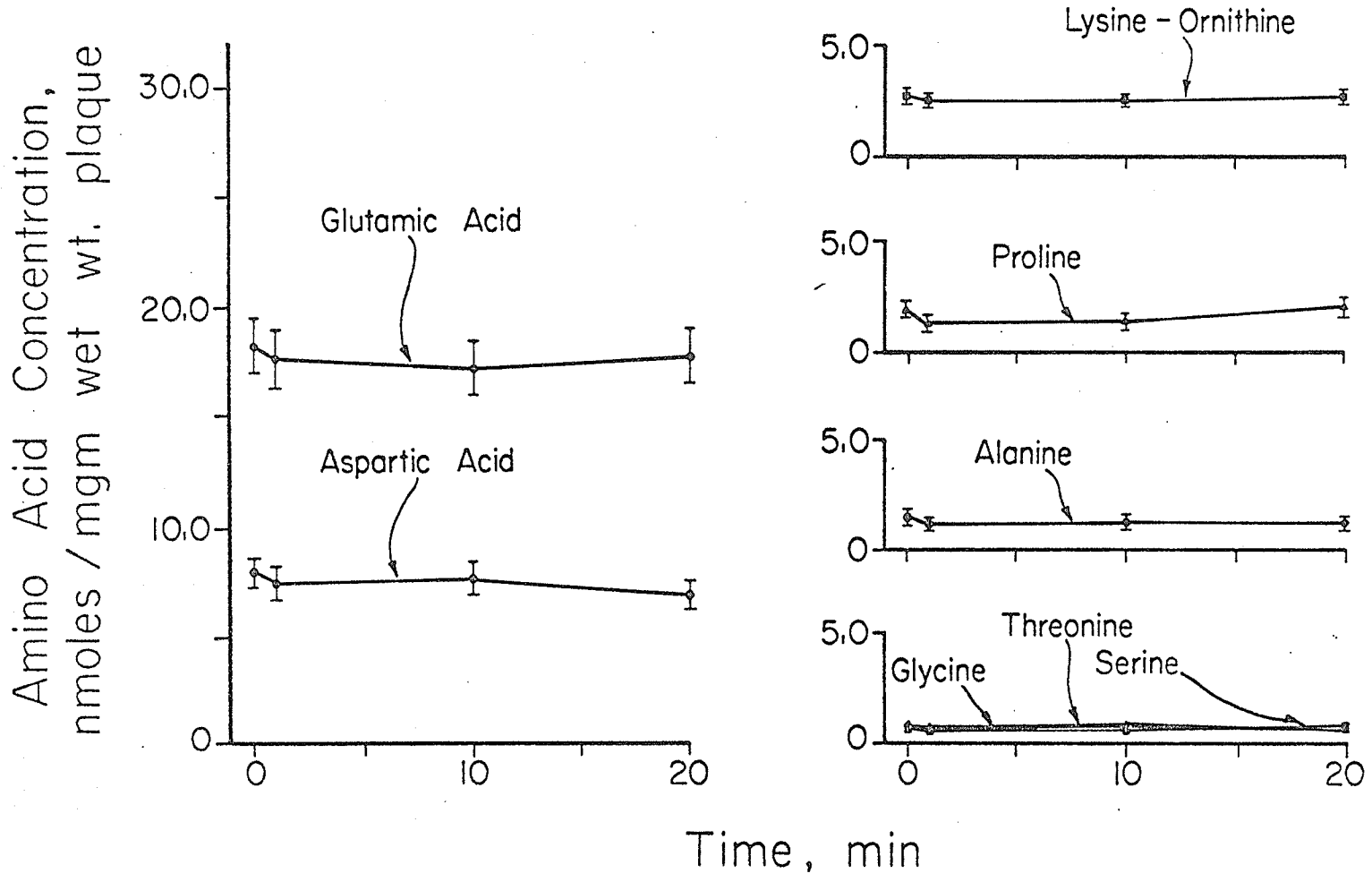


Fig. 5.4. Composition of the plaque free amino acid pool following a rinse with distilled water.

The increase in the size of the pool after exposure of the plaque to urea was due mainly to an increase in the concentration of aspartic acid and to a lesser extent to increases in glutamic acid and alanine (Fig. 5.5). The levels of lysine+ornithine, proline, threonine, serine and glycine were more or less unaffected by the rinse.

The change in the size of the pool following the glucose rinse was again due mainly to the two dicarboxylic amino acids (Fig. 5.6). Though both had fallen as early as 1 min. after the rinse, glutamic acid continued to decrease significantly during the remainder of the experimental period. The levels of the other amino acids either rose somewhat following the rinse (lysine+ornithine, alanine) or remained more or less unchanged (proline, threonine, serine, glycine).

The rise and fall in the amino acid pool following the rinse with urea plus glucose was due mainly to the behaviour of glutamic acid Fig. 5.7). Aspartic acid and proline behaved differently; their levels decreased slightly following the rinse. Lysine+ornithine, glycine, serine and threonine were not affected much by this rinse.

Alanine showed the most striking change. It sharply increased in concentration within the first 10 min. after the rinse and reached a concentration nearly four times its base-line level.

Effect of urea, glucose, urea plus glucose and distilled water on the pH of plaque.

The pH changes that occur under the conditions of these experiments are shown in Fig. 5.8. Following the urea rinse, the plaque pH abruptly rose, reached a maximum in 5 to 10 min. and then gradually fell towards base-line levels. The plaque pH with glucose showed a reverse pattern. When either distilled water or the urea-glucose rinse was used, the plaque pH changes were comparatively small. Both showed a slight fall

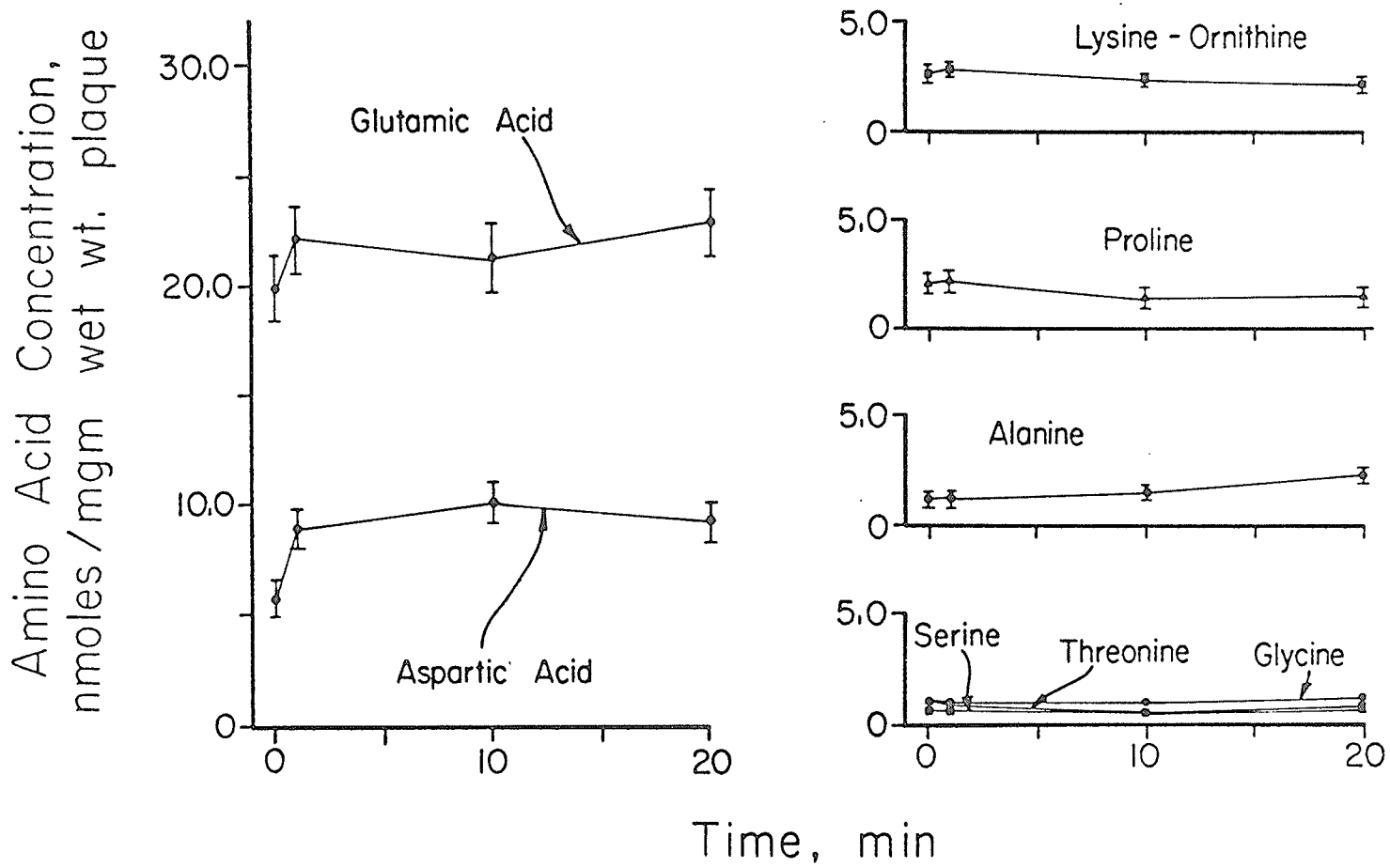


Fig. 5.5. Composition of the plaque free amino acid pool following a rinse with urea (0.28 M).

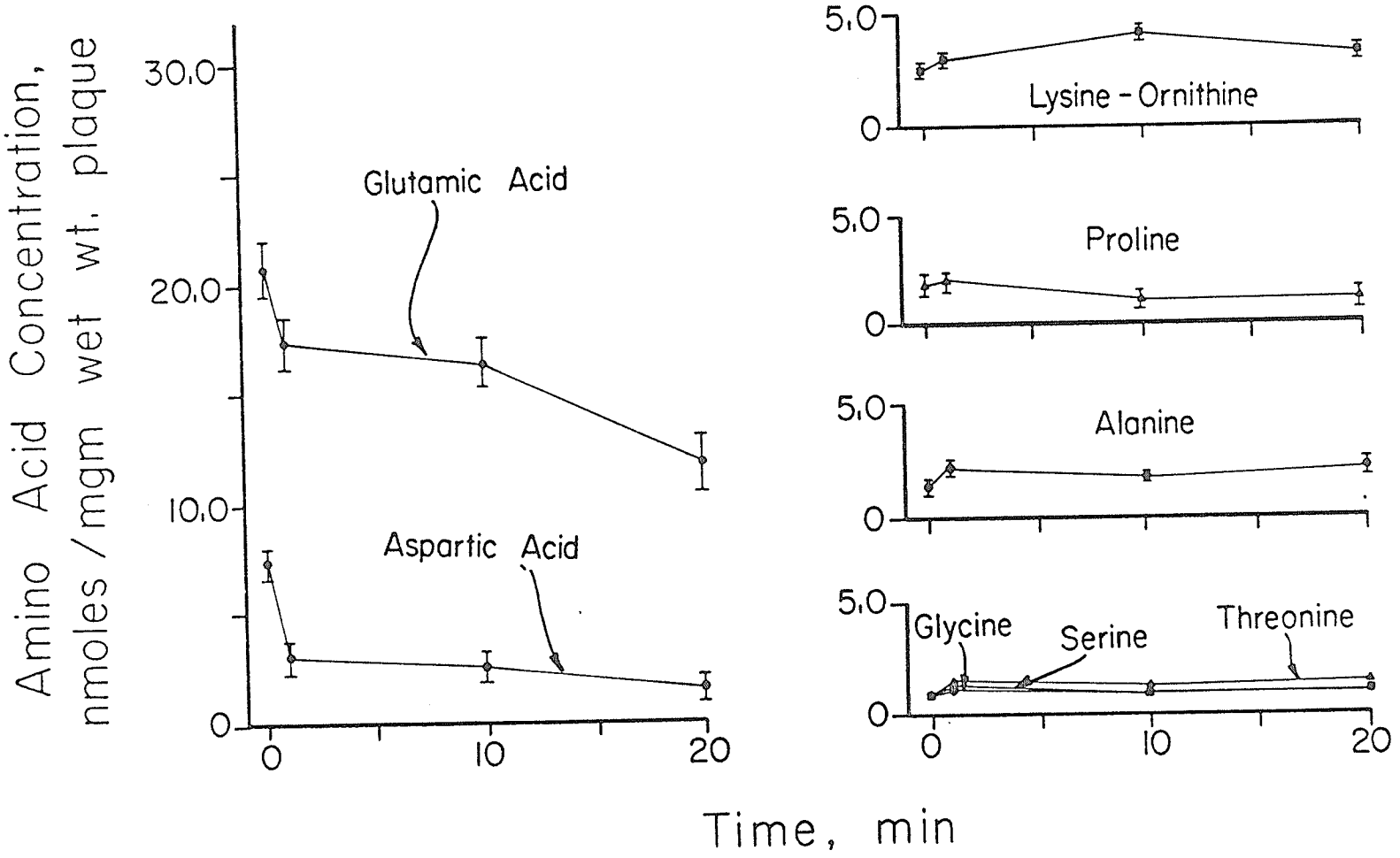


Fig. 5.6. Composition of the plaque free amino acid pool following a rinse with glucose (0.28 M)

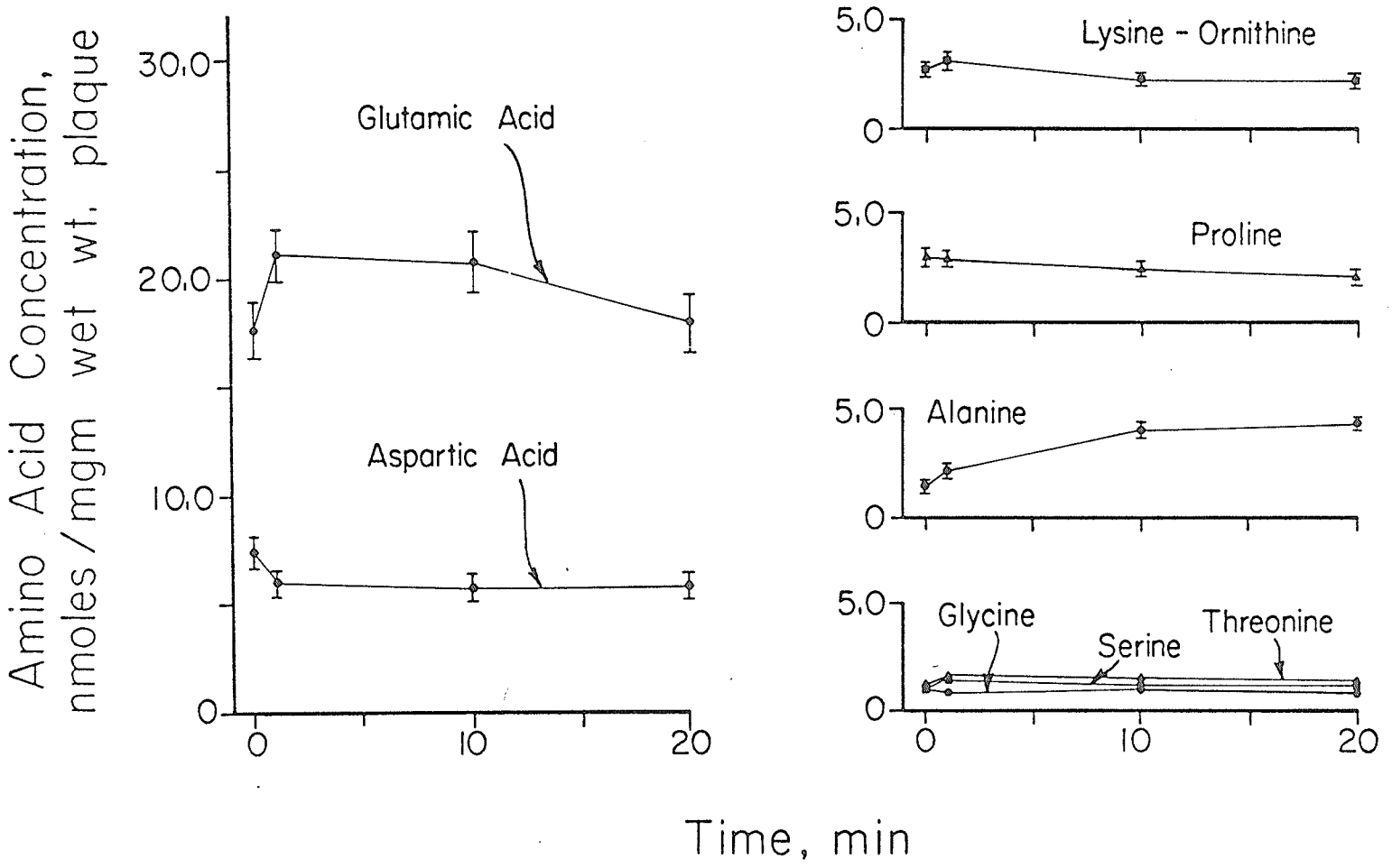


Fig. 5.7. Composition of the plaque free amino acid pool following a rinse with glucose plus urea (each 0.28 M)

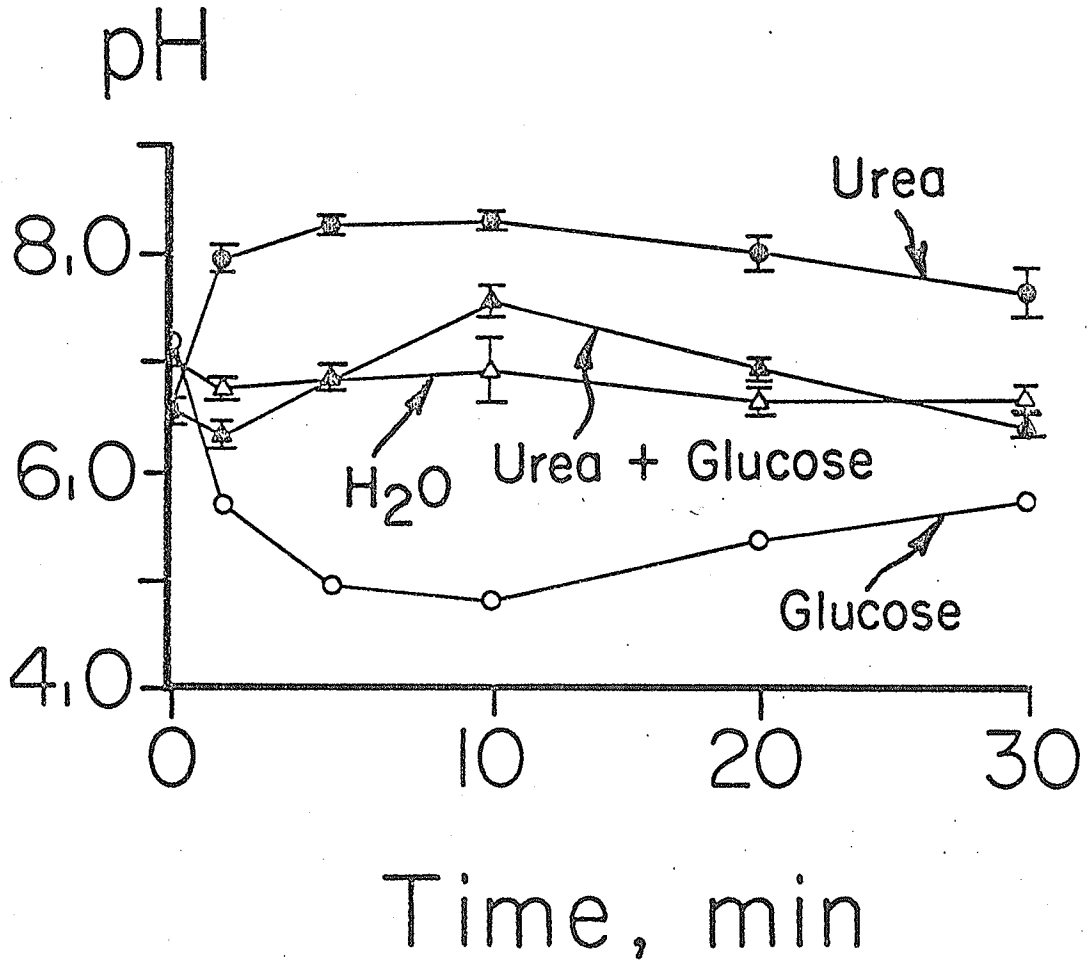


Fig. 5.8. Changes in pH following a rinse with either (i) urea (0.28 M) (ii) glucose (0.28 M), (iii) glucose plus urea (each 0.28 M) and (iv) distilled water. Each value represents the mean ± SEM of 3 experiments with either distilled water or glucose plus urea, 2 experiments with urea, and 1 experiment with glucose.

in pH at 1 min, a rise by five and ten min and then a gradual fall. These changes were greater with the urea plus glucose rinse than with distilled water. However, at no time was the pH with either the urea plus glucose rinse or the rinse with water significantly different.

Amino acid composition of hydrolysates of plaque bacteria

The amino acid composition of acid hydrolysates of plaque bacteria or of plaque matrix were decidedly different from the HCl extracts (Fig. 5.9). The major amino acids found in the bacterial hydrolysates were alanine, glutamic and aspartic acids, glycine, leucine and lysine. Methionine, ornithine, histidine and tyrosine were only present in small amounts while cystine could not be detected.

DISCUSSION

This study has shown that plaque *in situ* contains a pool of free amino acids which behaves like that of other cellular systems in that it is easily extracted with dilute HCl (HANCOCK, 1958) and its composition changes following exposure to a carbon or nitrogen source (DAWSON, 1965; TEMPEST *et al.*, 1970). Since all cells including bacteria contain a pool of free amino acids (HOLDEN, 1962), the plaque pool must include the intracellular amino acids of the plaque bacteria and in addition include those amino acids arising from the hydrolysis of cellular or matrix protein by extracellular proteases (CRITCHLEY, 1969).

Several factors suggest that most of the amino acids in the plaques analyzed in this study come from an intracellular pool. Firstly, the pattern of amino acids extracted was clearly different from the patterns found in hydrolysates of the cellular and acellular components of the plaque. Secondly, the high proportion of dicarboxylic amino acids with predominance of glutamic acid is characteristic of many microbial intracellular pools (HOLDEN, 1962; DAWSON, 1965; TEMPEST *et al.*, 1970;

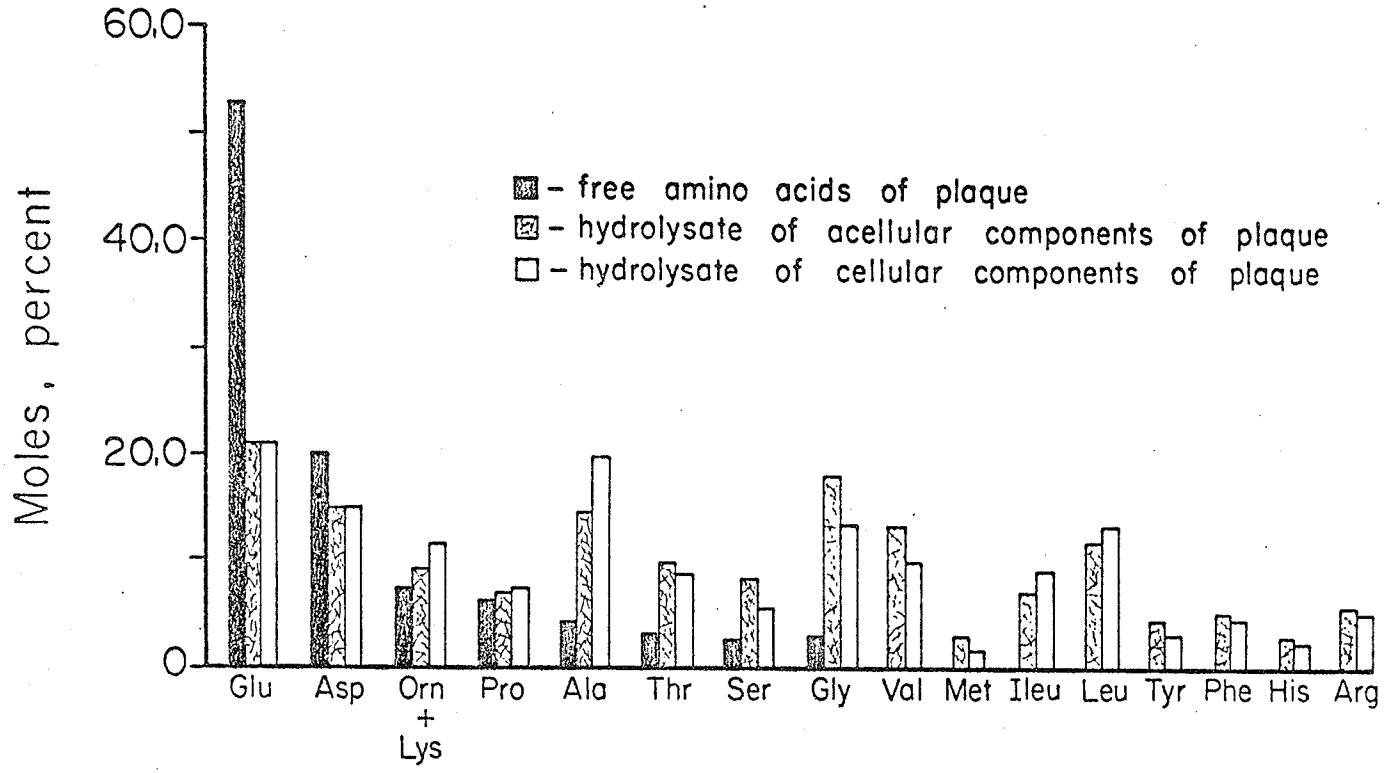


Fig. 5.9. Comparison between the composition of the plaque free amino acid pool, hydrolysates of the acellular components of plaque and hydrolysates of the cellular components of plaque. (Data for hydrolysates of the acellular components of plaque from, Silverman and Kleinberg, 1967a)

COWMAN et al., 1973). Thirdly, following the substrate rinses, the composition of the pool changed in a highly improbable manner to that expected had the alteration been caused by the degradation of matrix protein. Such degradation would have resulted in a parallel increase in virtually all amino acids; however, exposure to urea, glucose or both combined resulted in selective changes in the pool, mainly those amino acids involved in the primary pathways of amino acid metabolism. Finally, rinsing with water reduced the amino acids in the pool proportionately, whereas glucose and urea caused alterations of the pattern. This is apparent when the pool composition following the water rinse and the glucose+urea rinse are compared. While the changes in pH following these rinses were not too different, there were clearly differences in the composition of the plaque free amino acid pool.

The high concentrations of glutamic and aspartic acids in the plaque pool and their central role in cellular transamination reactions leading to the synthesis of other amino acids (LAMANNA and MALLETTE, 1965) suggests a similar role for these amino acids in the dental plaque. Previous results with sediment and the results of the present study with plaque suggest that alanine is the most important amino acid formed by this means.

The synthesis of alanine requires both a carbon source to provide pyruvate and a nitrogen source to provide an amino group. Since these conditions are best met when the plaque is exposed to glucose plus urea but not when it is exposed to either glucose or urea alone, it becomes clear why the concentration of alanine rises sharply under the former but not under the latter conditions. Perhaps the large increases in the concentration of aspartic and glutamic acids following the urea rinse is due to insufficient pyruvate to remove the amino groups. The decrease in aspartic and glutamic acids following the glucose rinse may be the result

of incorporation of some of these amino acids into protein, since the ATP generated during glucose degradation favours protein synthesis (LAMANNA and MALLETTE, 1965). It is obvious that tracer experiments, difficult as they may be to carry out with plaque in situ, would have to be performed to determine if these speculations are correct.

However, the large rise in alanine with the glucose plus urea rinse is particularly significant since similar conditions produced a similar increase in salivary sediment (BISWAS and KLEINBERG, 1967). In sediment, the rise was attributed to the diversion of pyruvate from acid forming pathways to the formation of alanine (BISWAS and KLEINBERG, 1967).

Besides providing evidence for urea and glucose being catabolized in a similar manner both in plaque in situ and in the salivary sediment system in vitro, the similarity in alanine behaviour in the two system provides additional evidence for the hypothesis that the catabolic pathways of glucose and urea which results in the formation of acid and base, are closely integrated.

CRITCHLEY (1969) found that aqueous extracts of plaque contained relatively high levels of alanine. He suggested that the alanine, along with the other amino acids in the extracts, is derived from proteolysis of plaque matrix proteins. Its accumulation, he proposed, was due to either its slow metabolism by the plaque bacteria or to this amino acid not being essential. An alternate possibility is that stored carbohydrate might have still been available along with salivary urea for the synthesis of alanine by transamination, since the plaque samples in this study were collected only two hours after eating.

Apparently, in endogenously formed bacterial pools, alanine is often found at relatively high levels and often accumulates in the medium (UMBARGER, 1969). Under these conditions excess alanine seems to be the result of the amount of pyruvate which accumulates during glycogen

catabolism provided that nitrogen and TPNH are available within the cell for reductive amination (UMBARGER, 1969). In the plaque, stored polysaccharide could supply the pyruvate, ammonia could supply the nitrogen (cf. CHAPTER II) and some glucose residues from glycogen metabolized via the hexose monophosphate shunt (SANDHAM and KLEINBERG, 1970a) could provide the TPNH.

The presence of mechanisms to produce large amounts of alanine may be important for the acid-base balance of plaque in situ. Alanine could be used for counteracting the acid formed from glucose. This might be accomplished in two ways. Firstly, increased formation of alanine would result in less acid being formed (either lactic, acetic or propionic) from pyruvate (SANDHAM and KLEINBERG, 1970a). Secondly, any alanine formed, if decarboxylated, would be converted to CO_2 and an amine. This, in turn, would favour acid neutralization and once glucose utilization and acid formation had ceased would favour the pH rising (KLEINBERG, 1970). Alanine might also be used to provide energy, since after deamination the pyruvate can be converted to acetic acid. If this should occur at alkaline pH loss of the ammonia by volatilization would mean that alanine would contribute to acid formation.

It is interesting to note that only small differences were apparent in the composition of the free amino acid pools of fasted plaques from the different dentition sites. This would be the case if the main determinant of the pool composition was the availability of salivary urea (see CHAPTER II). Furthermore, the differences that were apparent (i.e., tendency for glutamic and particularly aspartic acids to be higher in mandibular interproximal plaques (Fig. 5.2))are those one would expect with a greater availability of urea (cf. Fig. 5.5).

C H A P T E R V I

STUDIES ON THE AGGREGATION OF PLAQUE BACTERIA

During examination of the factors affecting the aggregation of the mixed bacteria found in dental plaque, SILVERMAN and KLEINBERG (1967 b) showed that maximum aggregation occurred when the pH was lowered below approximately 4 to 5. Either raising the ionic strength or adding divalent cations caused the pH of maximum aggregation to shift to higher values. This behaviour of the mixed plaque bacteria indicated that their cell surfaces were negatively charged above pH 4.0 to 5.0 (SILVERMAN and KLEINBERG, 1967 b).

The pH of the tooth surface can vary between about 6.0 and 7.5 in the absence of plaque (BRAWLEY, 1935; GROSSMAN and BRICKMAN, 1936; ERICSSON, 1949) and between 4.0 to 9.0 in its presence (KLEINBERG and JENKINS, 1964; KLEINBERG, 1967 b; De BOEVER et al., 1969). The ionization of a number of the chemical groups commonly found on bacterial surfaces (e.g. amino, carboxyl and phosphate groups) are markedly affected by such change in the pH (JAMES, 1965). Since their ionization would affect the bacterial surface charge, it should also affect their ability to adhere to surfaces or partake in aggregation phenomena. Moreover, such surface groups would be influenced by other ions found in the oral environment.

Studies concerning the role of streptococci in dental caries have established that differences exist in the ability of the various streptococci to adhere to oral surfaces (Van HOUTE, 1971; GIBBONS and Van HOUTE, 1971). These differences have been used to explain (i) the evidence that different bacterial types show a preference for certain surfaces when colonizing (GIBBONS and Van HOUTE, 1973) and (ii) the

hypothesis that specific organisms are responsible for the initiation of caries at different dentition sites (GIBBONS, 1968).

An earlier study has shown that the pH, ionic strength and calcium concentration markedly influence the aggregation behaviour of the mixed bacterial population of dental plaque (SILVERMAN and KLEINBERG, 1967 b). Because of the lack of similar information about the aggregation properties of the streptococci that have been investigated for their role in the dental caries process, the present study has examined the effects of these same variables on the aggregation behaviour of pure cultures of several strains of Strep. mutans, several strains of Strep. sanguis and one strain of Strep. salivarius. Experiments were also carried out to determine the aggregation behaviour of several mixed bacterial populations: (i) binary mixtures of pure cultures of the above streptococci (ii) bacterial fractions obtained from dental plaque using the technique of continuous particle electrophoresis, and finally (iii) bacteria harvested after incubation of enamel or gingival plaque in brain heart infusion broth for 24 hours.

METHODS AND MATERIALS

Preparation of microorganisms

(a) Pure cultures of various oral streptococci

The oral streptococci studied were: Strep. mutans strains AHT, BHT, GS-5, OMZ 176, Ingbritt, PS-14, 6715 (obtained from H. J. Sandham, Toronto, Canada); Strep. sanguis strains, ATCC 10558, 10557, and 10556 (obtained from T. F. McNamara, Morris Plains, N. J., U. S. A.) and Strep. salivarius strain ATCC 25975 (obtained from I. R. Hamilton, Winnipeg, Canada).

Stock cultures were grown for 18 hours in Brain-Heart-Infusion broth and stored at -20°C. The stability and purity of the stock sus-

pensions were checked at monthly intervals and fresh cultures were used for each experiment.

Each microorganism was grown anaerobically at 37°C in Brain-Heart-Infusion broth (Difco) under an atmosphere of 95 percent nitrogen and 5 percent carbon dioxide. Cells, either in the exponential or the stationary growth phase, were harvested by centrifugation (13,400g for 20 minutes at 4°C) and washed three times either with distilled water at pH 7.0 or with 0.1 N NaOH before re-suspending in fresh wash fluid at a cell concentration of 10 percent (V/V).

(b) Bacteria fractionated from dental plaque

Plaque was removed with a stainless steel spatula from the teeth of five to ten subjects who had not cleaned their teeth for three days nor eaten for 12 hours prior to plaque collection. Collection was always carried out on the fourth day between 9:00 and 10:00 a.m. Each portion of plaque upon removal, was immediately dispersed in cold 0.1 N NaOH; this separated the bulk of the bacteria from the plaque matrix (SILVERMAN and KLEINBERG, 1967 a). The plaque cells were then washed three times and stored in veronal buffer (0.001 M, pH 8.6 at 4°C) for subsequent electrophoretic separation on a continuous particle electrophoresis (CPE) apparatus (Beckman). Storage of the bacteria in the buffer was never longer than 18 hours. Immediately prior to electrophoresis, the plaque bacteria were rewashed and re-suspended in fresh buffer.

The bacterial concentration in each sample applied to the CPE apparatus (less than 1 percent V/V) was one that produced a visible, stable, thread-like, zero-voltage reference band (SINSABAUGH and NORMORE, 1971). Typical operational parameters were: curtain flow

rate 15 ml/minute, sample injection flow rate 20 µl/ minute and applied voltage gradient 55 volts/cm. The potential was applied once the zero-voltage reference band was established. After band equilibration (three minutes), collection of the effluent from each of the 48 outlets was begun.

The absorbance of an aliquot (200 µl) from each of the 48 samples was measured spectrophotometrically at 550 nm. The bacteria in each sample were then harvested by centrifugation (12,000g for 30 minutes at $4^{o}C$) and stained with crystal violet for examination by light microscopy.

(c) Bacteria in plaque and gingival scrapings incubated in Brain Heart Infusion broth

Using a sterile spatula, three day old plaque (about 0.5 mg wet wt) was removed first from the labial surfaces of the maxillary incisors and then from the corresponding attached gingivae of three subjects (same conditions of collection as in Section b). The enamel and gingival plaque material were each immediately transferred and dispersed in Brain Heart Infusion broth (Difco) and then incubated aerobically or anaerobically (95 percent N_2, 5 percent CO_2) for 24 hours at $37^{o}C$ in small tubes (13 x 100 mm, screw cap; Kimax). The contents of each tube were then transferred to 100 ml of fresh medium and incubated for a further 18 hours. The organisms were harvested by centrifugation (13,400g for 20 minutes at $4^{o}C$), washed three times with phosphate buffered saline (pH 7.0), three times with 0.1 N NaOH and then suspended in 0.1 N NaOH at a final cell concentration of 10 percent (V/V).

Method for determining aggregation patterns

Aggregation of bacteria was determined turbidometrically (cf.

RYAN and KOLIN, 1964). An aliquot (20 µl) of stock suspension was centrifuged (11,000xg, Beckman Microfuge for 30 seconds); the supernatant was carefully removed by suction and the pellet was resuspended in 25 µl 0.1 N NaOH. The suspension was transferred with 2 ml of water to a cuvette (10 x 10 mm) positioned in a modified cell compartment (Fig. 6.1) of a spectrophotometer (Beckman Model 1098). A Teflon coated stirring bar was inserted into the cuvette and rotated at approximately 140 rpm with a magnetic stirrer located beneath the cell compartment. A glass pH electrode, KCl salt bridge and a fine polyethylene delivery tube from a micro-burette (0.25 ml; KONTES, New Jersey) were inserted into the cuvette and positioned so as not to interfere with the light path of the instrument. After five minutes of equilibration, the pH and O.D. at 700 nm were measured. The sample was then titrated with HCl (0.1 or 0.05 µl additions of 1.0 N at one minute intervals) between about pH 10.0 and 2.0 and the pH and O.D. read immediately prior to each addition.

To check the relation between the O.D. reading and the appearance of a suspension (3 ml) during its titration, aliquots (20 µl) were transferred to a hemocytometer, viewed under phase-contrast microscopy, and photographed using a Zeiss Photomicroscope II.

Aggregation experiments

(a) Aggregation of pure cultures

The aggregation behaviour of pure cultures of each of the following: Strep. mutans strains AHT, BHT, Ingbritt, PS-14, OMZ 176, 6715, and GS-5; Strep. sanguis strains ATCC 10556, 10557, and 10558, and Strep. salivarius ATCC strain 25975, was examined. Each strain was harvested during the exponential growth phase, and then washed (three

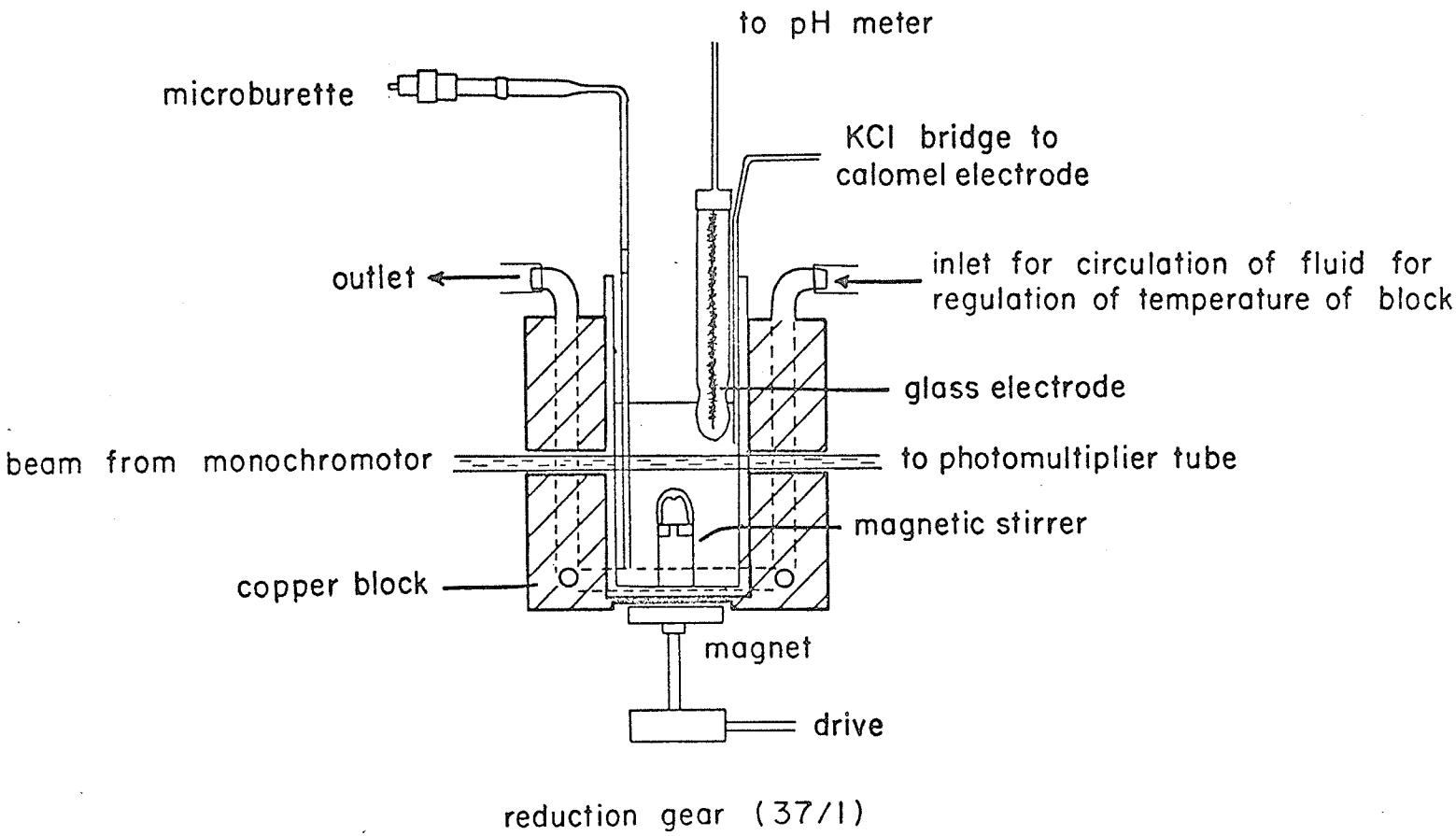


Fig. 6.1. Schematic diagram of the cuvette region of the spectrophotometric-pH electrode assembly used to determine the aggregation patterns of bacterial suspensions.

times) and suspended in distilled water previously adjusted to pH 7.0 with NaOH.

(b) The effect of (i) harvesting during exponential or stationary growth phase and (ii) washing with cold 0.1 NaOH on aggregation

Since the growth phase of the bacteria involved in plaque formation is uncertain and since separation of the bacteria from plaque is accomplished by exposure to dilute NaOH, several strains of streptococci were examined for the effects of these parameters on their aggregation. The microorganisms examined were Strep. mutans strains BHT, AHT, and Ingbritt and Strep. salivarius.

Each strain was cultured anaerobically, harvested either in the exponential or stationary growth phase and then washed (three times) and suspended in either 0.1 N NaOH or distilled water (pH 7.0) prior to the testing of their aggregation behaviour.

(c) Effect of ionic strength (KCl) and calcium on aggregation patterns of pure cultures

The cells listed in section (a) were retested but in the presence of $CaCl_2$ (0.001 M or 0.004 M; Baker analyzed) or KCl (0.003 M or 0.012 M; Baker analyzed).

(d) Aggregation patterns of binary mixtures of pure cultures

In these experiments the streptococcal strains selected for combination were those with markedly different aggregation patterns. The combinations examined were (i) Strep. mutans AHT + Strep. salivarius; Strep. mutans BHT + Strep. sanguis 10558 (organisms were harvested in the exponential growth phase, water washed and aggregation determined in distilled water); (ii) Strep. mutans AHT + Strep. mutans PS-14 (organisms were harvested in the exponential growth phase, water washed

and aggregation determined in 1 mM $CaCl_2$); (iii) Strep. mutans BHT + Strep. mutans GS-5; Strep. mutans BHT + Strep. salivarius (organisms were harvested in the stationary growth phase, washed with 0.1 N NaOH, and aggregation determined in distilled water). All microorganisms were tested in 1:1 ratio. Strep. mutans BHT + Strep. salivarius were also tested in 3:1 ratio.

(e) Aggregation patterns of unfractionated plaque cells and cells fractionated by electrophoresis

Unfractionated plaque cells and three fractions obtained by electrophoresis which provided sufficient material for study were washed and suspended in distilled water (1 ml). Aliquots or the whole suspension were transferred to the cuvette of the spectrophotometer after adding 25 µl 0.1 N NaOH and using sufficient distilled water to bring the volume within the cuvette to 2 ml. The size of aliquot was one that gave an initial O.D. reading approximately equal to 0.08.

(f) Aggregation patterns of anaerobically or aerobically cultured tooth and gingival scrapings

The cultures from both the tooth and gingival scrapings whether grown anaerobically or aerobically contained predominantly Gram +ve cocci with some Gram +ve rods. Aliquots (20 µl) of the stock suspensions of each were transferred to the spectrophotometer cuvette as before and the aggregation patterns determined.

RESULTS

Relation between O.D. measurements and cellular aggregation

The relation between optical density and the microscopic appearance of a suspension of Strep. salivarius is shown in Fig. 6.2. Chains and single cocci were apparent in the initial suspension (approx.

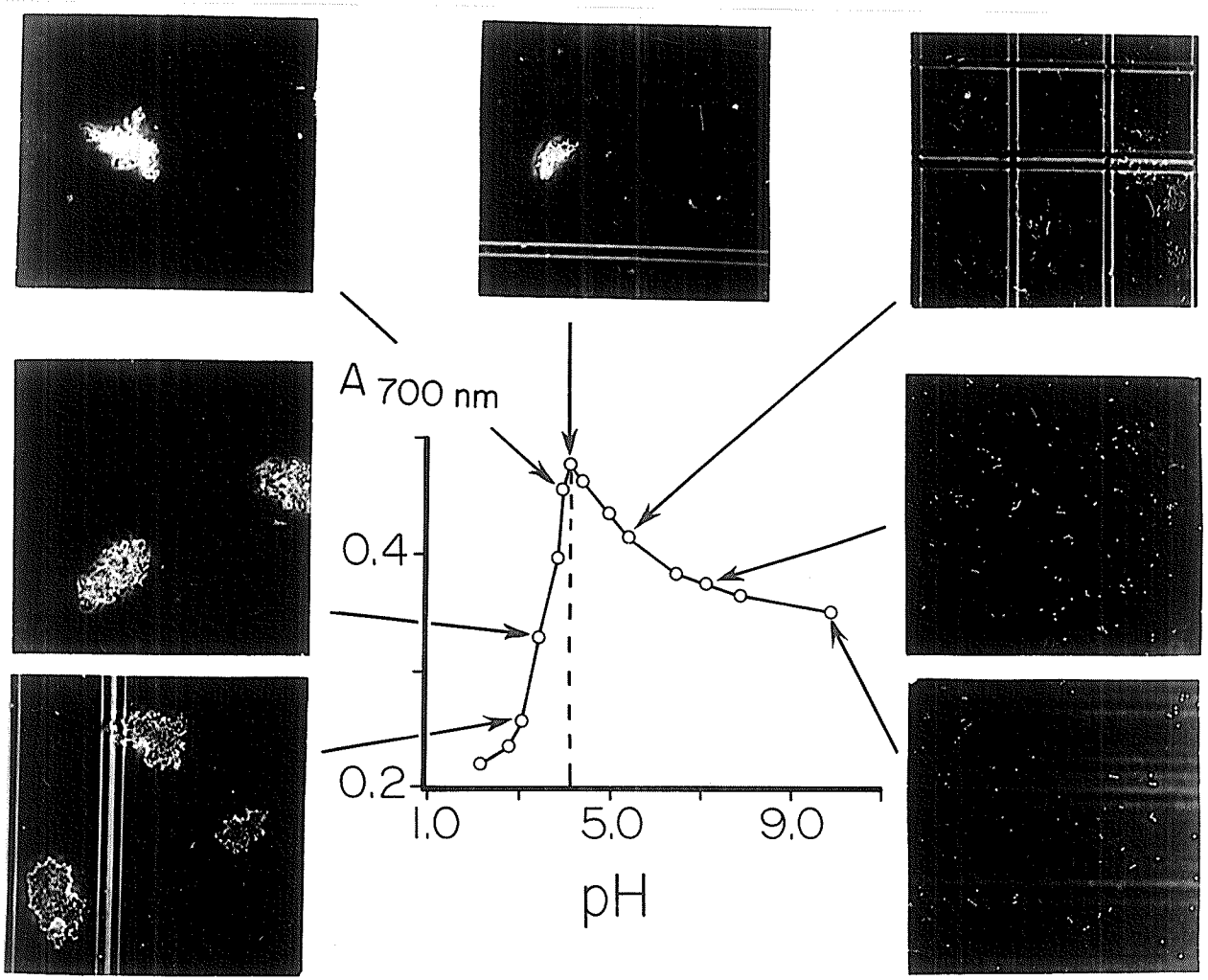


Fig. 6.2. The effect of pH on the relationship between optical density and the microscopic appearance of a suspension of Strep. salivarius.

pH 10); as the pH was decreased between approximately 10 and 5 occasional small clumps appeared. Upon further decrease in the pH, the O.D. reached a maximum and large irregular aggregates could be seen. The O.D. then showed a comparatively rapid decrease below the O.D. maximum which corresponded to the formation of progressively larger clumps. Thus, massive aggregation begins at the pH at which the O.D. reaches its maximum; below this pH, the aggregation continues and corresponds to a continuous decrease in the O.D.

Varying the suspension concentration, while increasing both the initial O.D. and the magnitude of the O.D. changes had little effect on the aggregation pattern or on the pH at which maximum aggregation began (Fig. 6.3).

Aggregation patterns of pure cultures of oral streptococci

The aggregation curves of the 11 streptococci examined were of three types (Fig. 6.4 and Table VI.1):

I - the O.D. showed little change between pH 10 and 7.5 but below this range, the O.D. rose rapidly to a maximum and was followed by an abrupt fall. Organisms showing type I aggregation were Strep. mutans strains PS-14 and GS-5, Strep. sanguis strains 10556 and 10557 and Strep. salivarius. The pH of maximum aggregation was between pH 3.5 and 4.3.

II - the O.D. changed in the same manner as the Type I curve except that the O.D. showed a plateau about 1.0 to 1.5 pH units above the pH of maximum aggregation. Organisms showing this type of aggregation pattern were Strep. mutans Ingbritt and Strep. sanguis 10558. The pH of maximum aggregation was between pH 3.5 and 3.6.

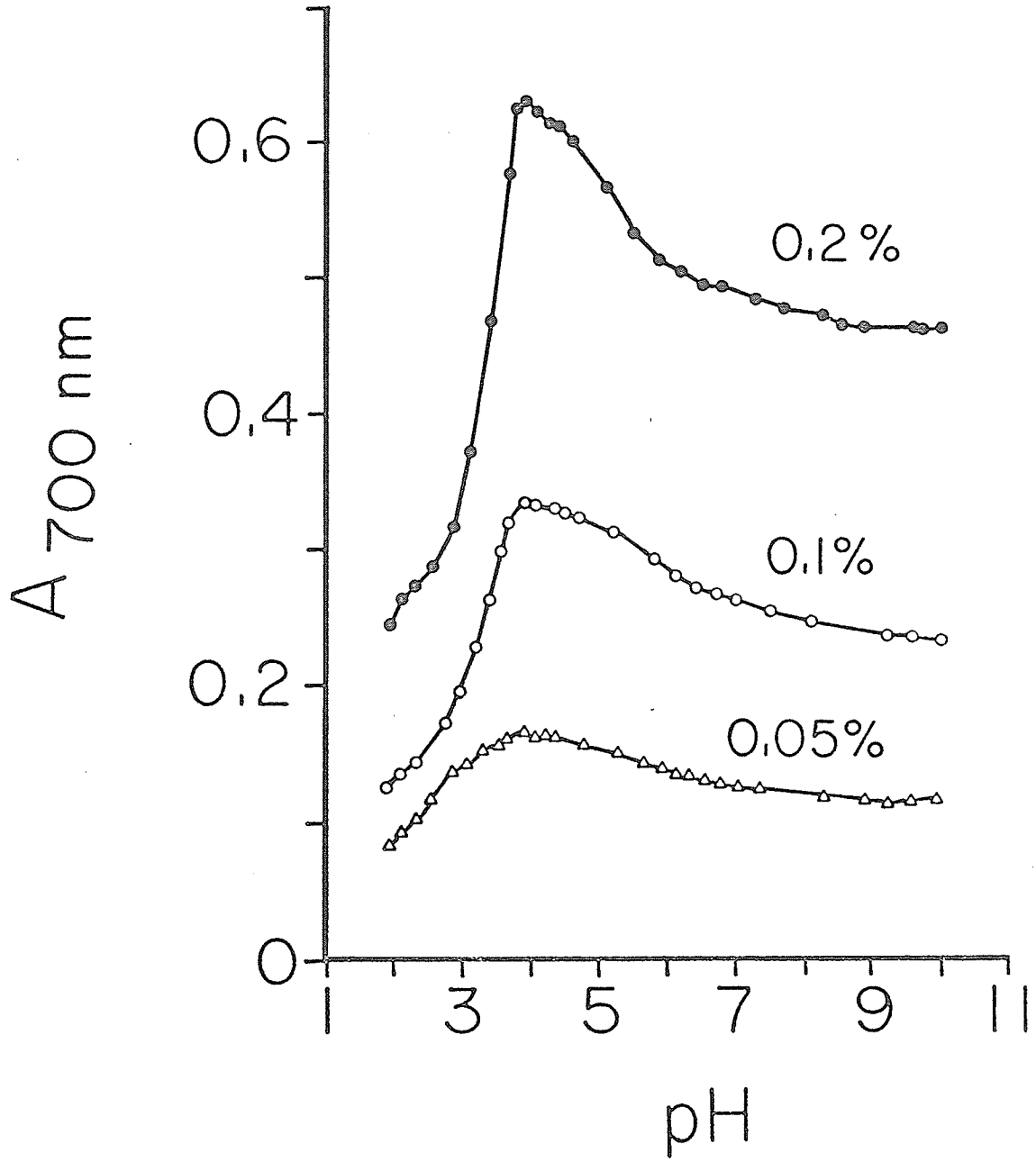


Fig. 6.3 Comparison of the aggregation patterns of suspensions of Strep. salivarius at varying cell concentrations (0.05, 0.10 and 0.20 percent (V/V)).

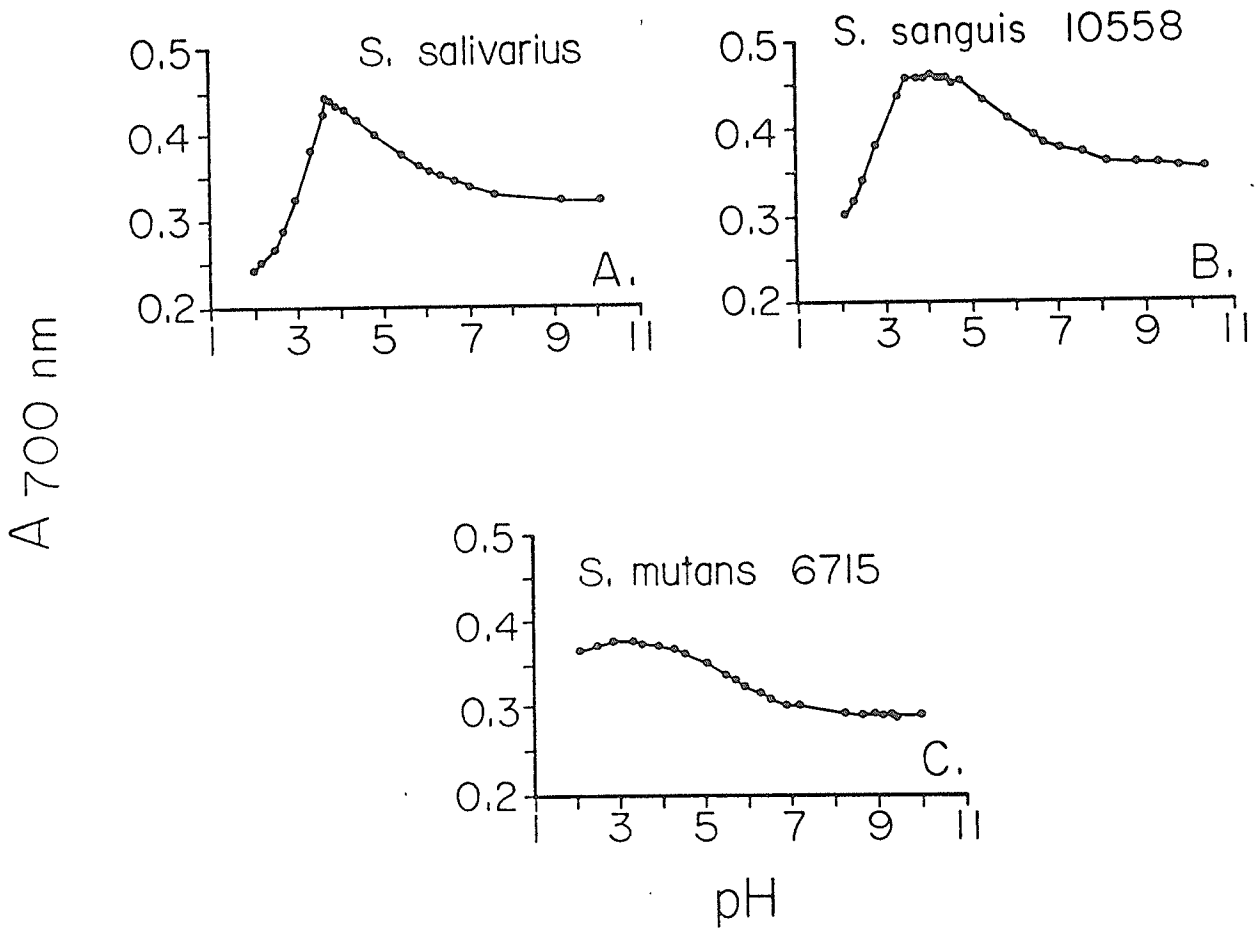


Fig. 6.4. The three typical aggregation patterns exhibited by anaerobically cultured streptococci harvested in the exponential growth phase and washed with distilled water (pH 7.0) A. Strep salivarius (Type I), B. Strep. sanguis 10558 (Type II) and C. Strep mutans 6715 (Type III).

Table VI.1. The pH of maximum aggregation of pure cultures of Streptococci[(a)].

Organism	Type*	N**	MA pH***
Strep. sanguis 10557	I	3	4.31 ± 0.08
Strep. sanguis 10556	I	6	4.01 ± 0.03
Strep. mutans PS-14	I	3	3.90 ± 0.09
Strep. salivarius	I	5	3.82 ± 0.05
Strep. mutans GS-5	I	3	3.49 ± 0.08
Strep. mutans Ingbritt	II	5	3.64 ± 0.03
Strep. sanguis 10558	II	4	3.50 ± 0.09
Strep. mutans BHT	III	6	3.35 ± 0.07
Strep. mutans 6715	III	4	2.97 ± 0.10
Strep. mutans AHT	III	3	2.85 ± 0.14
Strep. mutans OMZ 176	III	2	2.47 ± 0.10

(a) bacteria harvested in exponential growth phase; washed with distilled water; media in which aggregation was determined was distilled water.

* type of aggregation pattern as shown in Fig. 6.4

** number of fresh cultures examined (in duplicate).

*** pH of maximum aggregation ± standard error of mean.

III - the O.D. showed a more gradual rise and fall with decrease in the pH; also, the pH of maximum aggregation occurred at a lower pH than in the Type I and II curves. Organisms showing this type of aggregation curve were Strep. mutans strains AHT, BHT, 6715 and OMZ 176. AHT and BHT occasionally exhibited a slight fall in O.D. (approx. 0.015 O.D. units) as the pH was decreased from about pH 10.0 to about 7.0. The pH of maximum aggregation for these organisms was in the range pH 2.4 to 3.4.

The effect of growth phase and washing with 0.1 N NaOH on the aggregation patterns of pure cultures

The effects of the growth phase in which the organisms were harvested and the washing solutions used (water or 0.1 N NaOH) on the aggregation patterns of several microorganisms are shown in Figs. 6.5 and 6.6.

The aggregation patterns of Strep. mutans strains AHT and BHT were affected by both the time of harvesting and the solution in which the harvested cells were washed. The cells harvested in the stationary phase showed a sharp O.D. maximum at low pH which did not occur when they were harvested in the exponential growth phase. Washing the stationary cells of the AHT and BHT strains with 0.1 N NaOH instead of distilled water lowered the pH slightly at which this peak occurred. Strep. mutans strain Ingbritt was only affected by the wash solution. Harvesting of these cells in either growth phase resulted in an O.D. maximum with a lower pH when the cells had been washed with 0.1 N NaOH than with distilled water. Strep. salivarius, on the other hand, was relatively unaffected by either the growth phase or the wash solution, though NaOH washed cells did tend to show a somewhat sharper O.D. maximum.

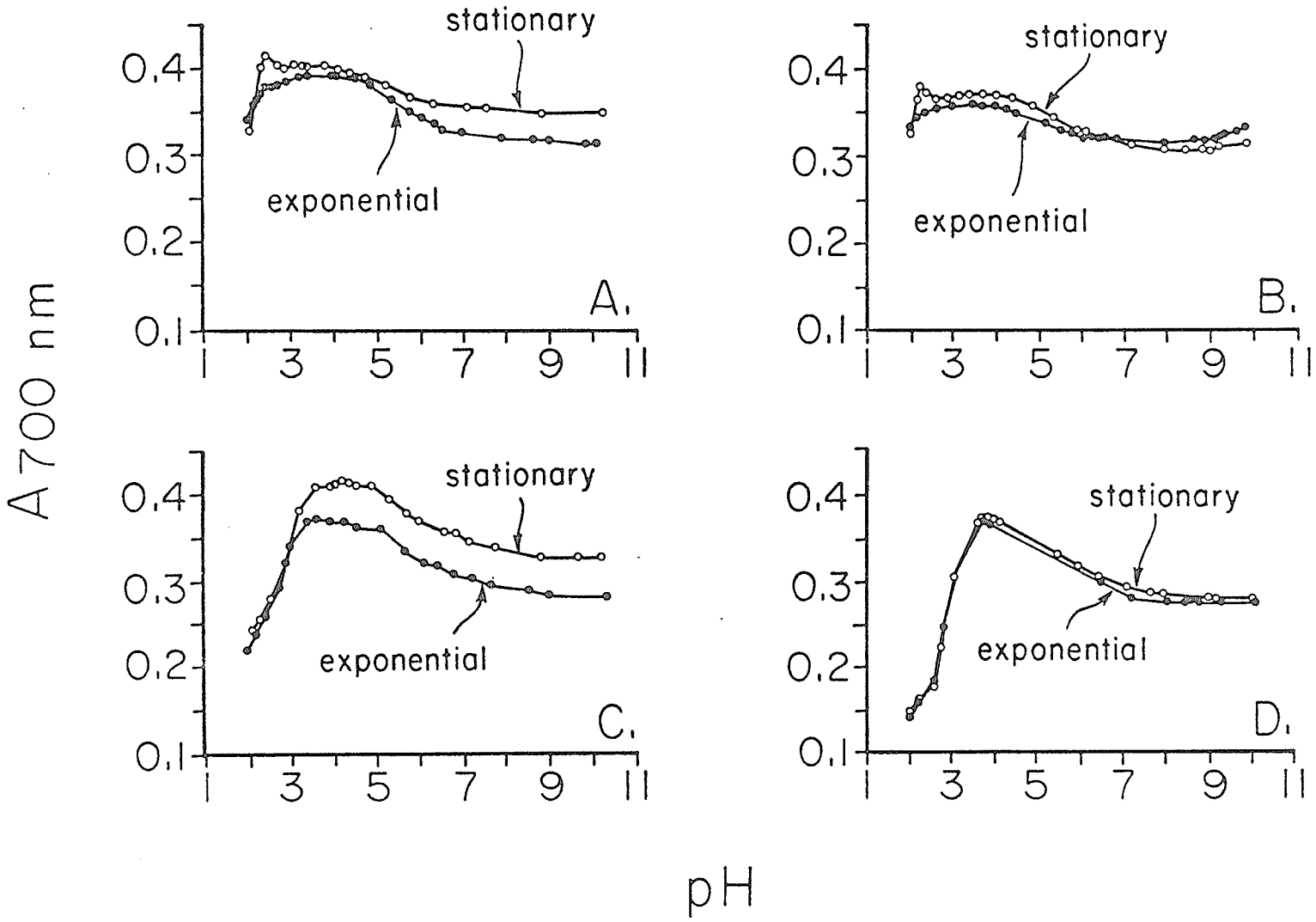


Fig. 6.5. The effect of harvesting in either the exponential or stationary growth phase on the aggregation pattern of pure cultures of (A) Strep mutans BHT, (B) Strep mutans AHT, (C) Strep mutans Ingbritt and (D) Strep salivarius.

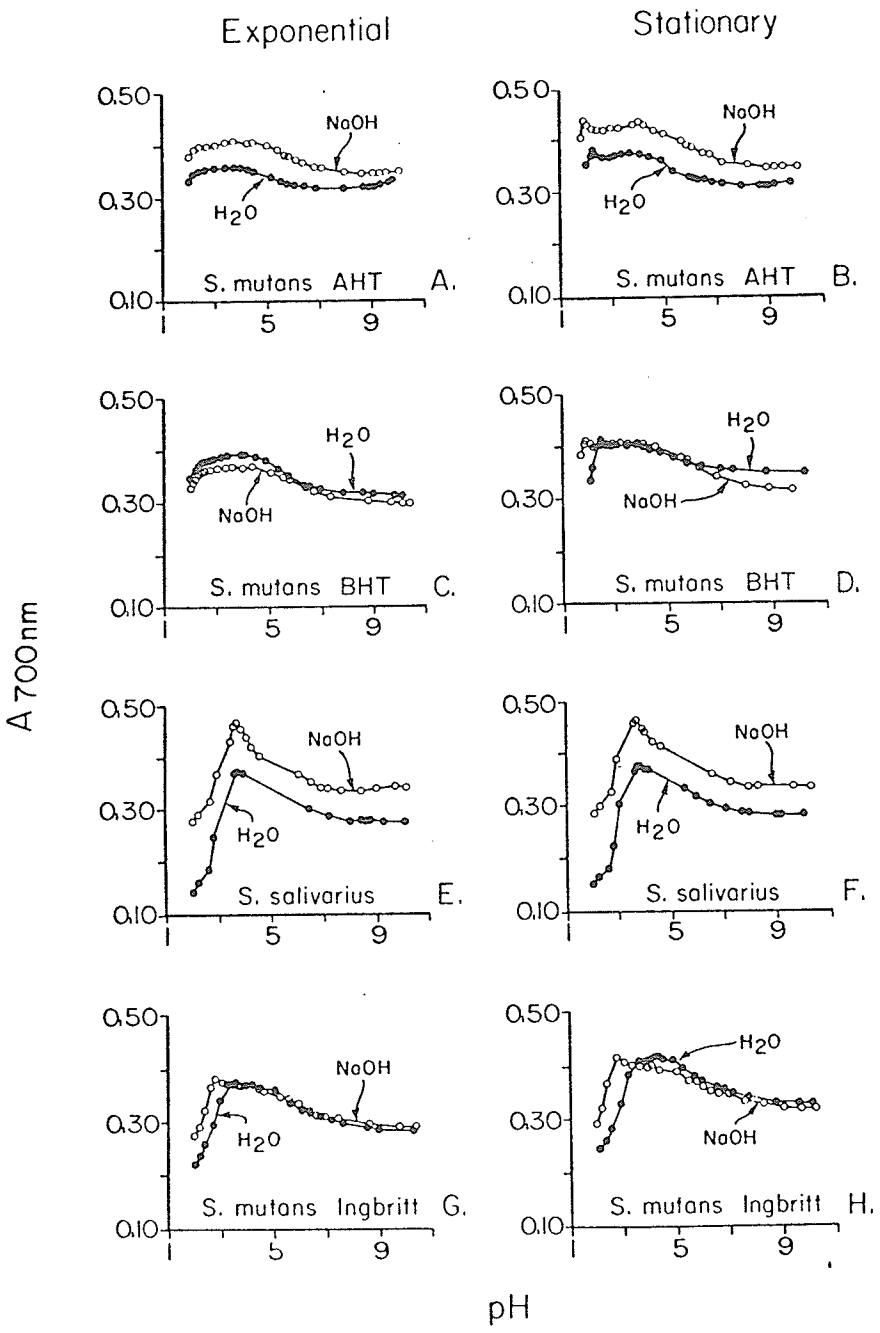


Fig. 6.6. The effect of washing with distilled water (pH 7.0) or 0.1 N NaOH on the aggregation patterns of pure cultures of streptococci harvested either in the exponential or stationary growth phase.

Effect of ionic strength and calcium on the aggregation patterns of pure cultures

For all eleven streptococci examined, the pH of maximum aggregation was higher with potassium chloride than with distilled water. At constant ionic strength, calcium chloride favoured a higher maximum than potassium chloride. With calcium, the O.D. was generally higher initially and generally showed a small decrease before rising to a maximum. The typical types of aggregation patterns under these conditions are shown in Figs. 6.7, 6.8 and 6.9.

The shift in the pH of maximum O.D. with either increased ionic strength or calcium was least for Strep. mutans strains AHT, BHT, 6715, OMZ 176 and GS-5, Strep. sanguis 10556 and Strep. salivarius (Figs. 6.7 and 6.8). KCl had either no effect or shifted the pH about 0.3 pH units higher than with distilled H_2O. With 0.001 M calcium, the pH shift was about 0.5 pH units higher; with 0.004 M calcium, depending on the organism, the shift was either slightly more (Strep. mutans BHT, AHT, 6715, OMZ 176) or slightly less (Strep. sanguis 10556, Strep. mutans GS-5 and Strep. salivarius) than that with 0.001 M calcium.

In contrast, the shift in the pH of maximum aggregation with either increased ionic strength or calcium was much greater for Strep. mutans strains PS-14 and Ingbritt, and Strep. sanguis strains 10558 and 10557 (Fig. 6.9). The largest shifts were consistently found with 0.001 M calcium and with Strep. mutans Ingbritt, PS-14 and Strep. sanguis 10558. The pH of maximum aggregation under these conditions for these organisms occurred between pH 7 to 8; that for Strep. sanguis 10557 occurred at about pH 6.0. With 0.004 M calcium, the rise to the maximum O.D. was reduced and the pH of maximum aggregation usually

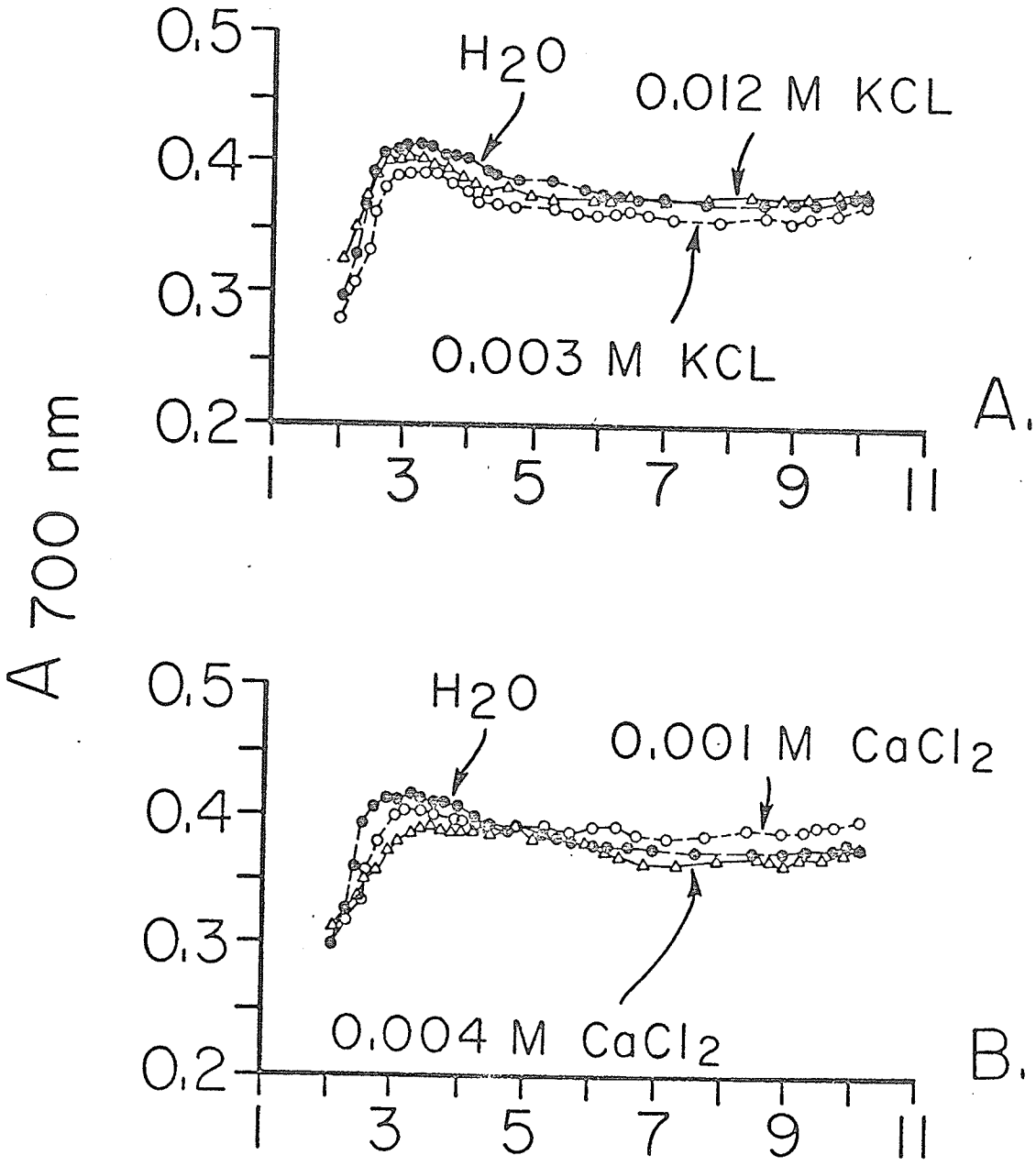


Fig. 6.7. Effect of KCl and $CaCl_2$ at similar ionic strengths, on the aggregation patterns of Strep mutans BHT.

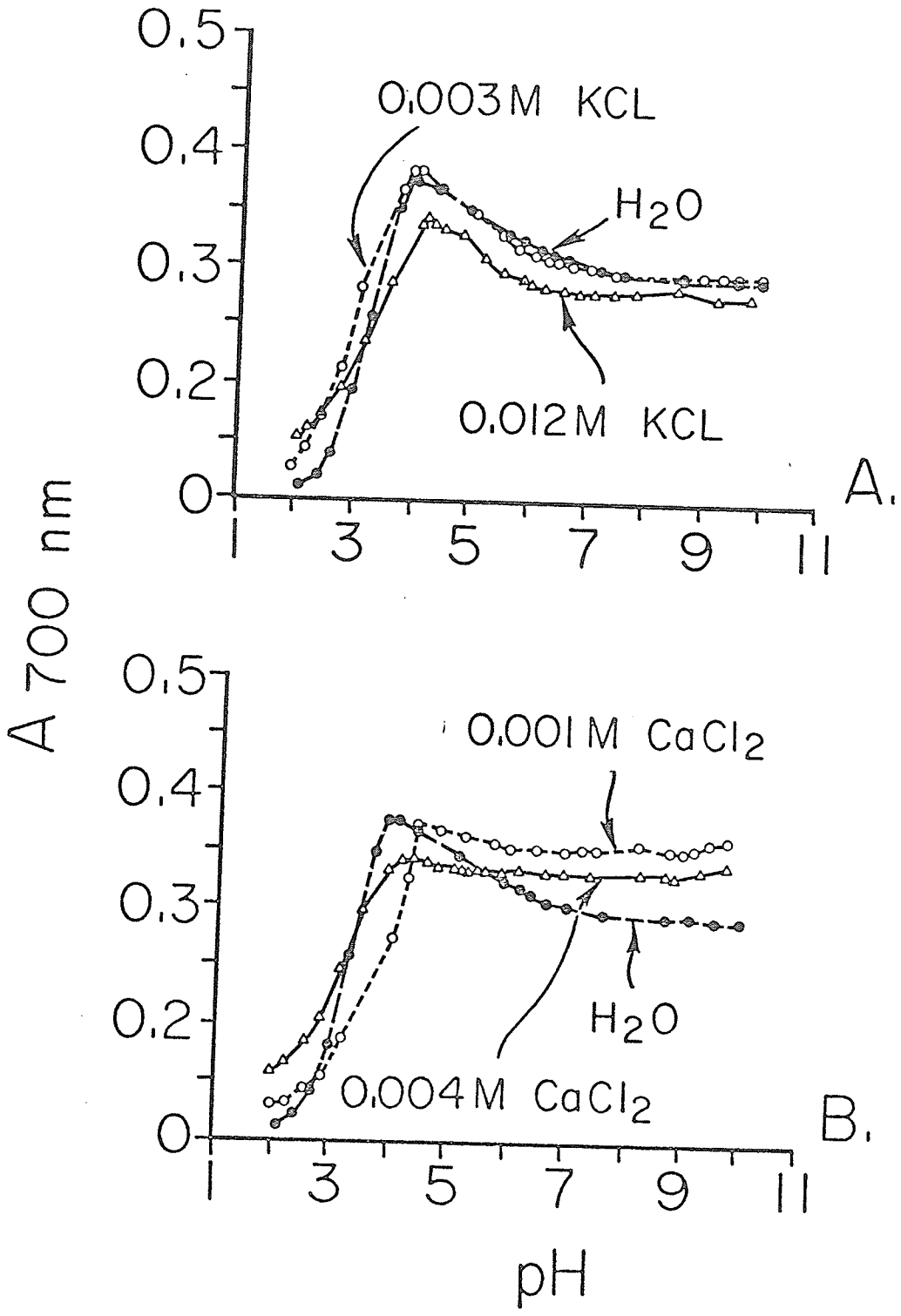


Fig. 6.8. Effect of KCl and $CaCl_2$ at similar ionic strengths on the aggregation patterns of Strep. salivarius.

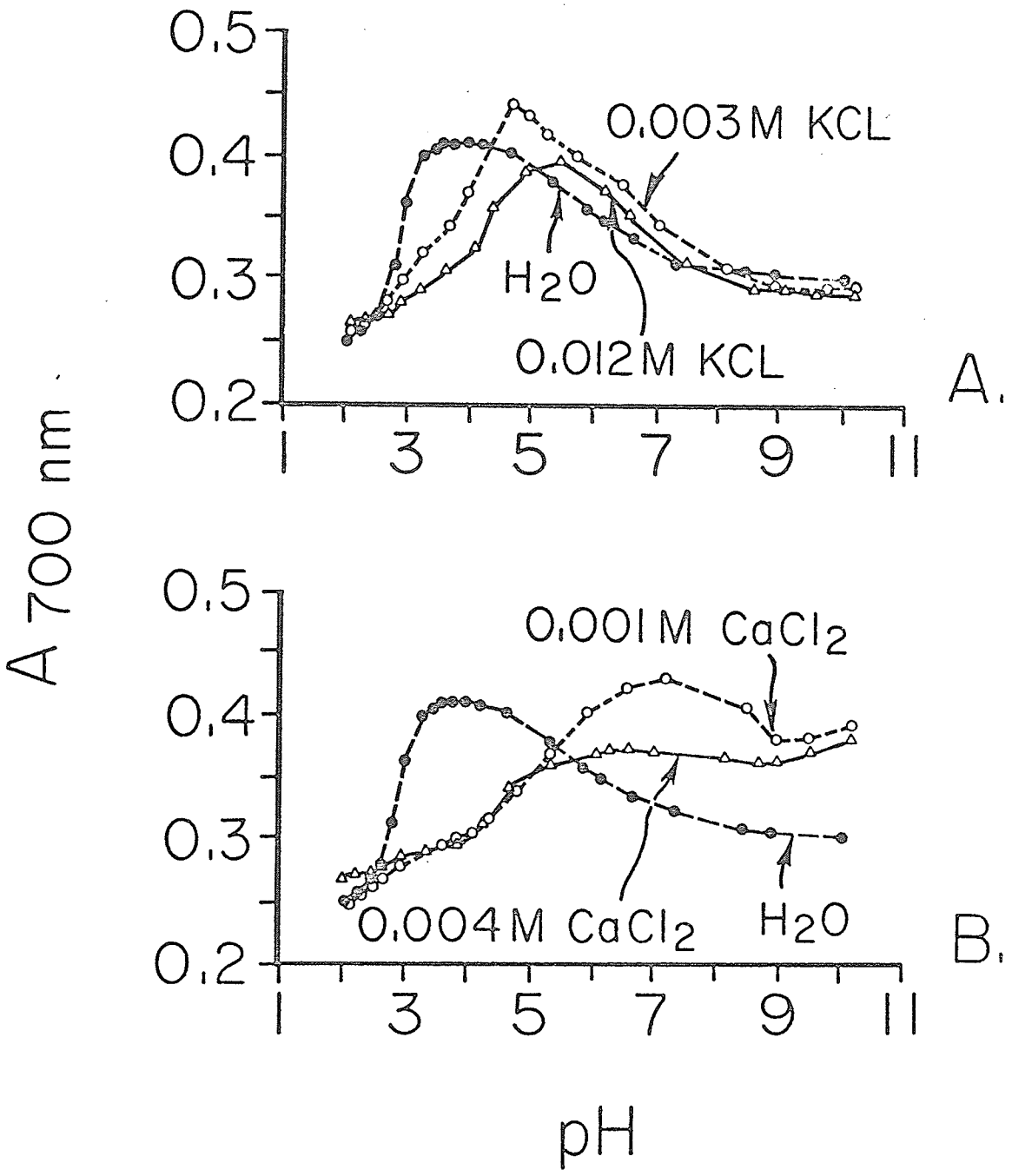


Fig. 6.9. Effect of KCl and $CaCl_2$ at similar ionic strengths, on the aggregation patterns of Strep mutans Ingbritt

occurred at a slightly lower pH than with 0.001 M. calcium.

Binary mixtures of pure cultures

In all of the binary mixtures tested (Figs. 6.10, 6.11, 6.12), the pattern of aggregation was most similar to that of the organism, within the mixture, which by itself had the higher pH of maximum aggregation (even when this organism constituted only 33.3 percent (V/V) of the suspension; Fig. 6.11). Conversely, the greatest difference in the patterns of aggregation was between the binary mixtures at pH levels more acid than their pH of maximum aggregation and the organism, within the mixture, which had the lowest pH of maximum aggregation.

Electrophoretic behaviour of the bacteria isolated from dental plaque

The bulk of the bacteria were attracted towards the anode (Fig. 6.13). Although distinct banding was not visible in the electrophoresis cell, a distribution of morphological types was apparent in the fractions collected (Fig. 6.14). Cocci and short rods showed least electrophoretic mobility; darkly staining "diplococci" showed moderate mobility; whereas, closer to the anode, filamentous and coccal forms predominated. The fraction showing the greatest mobility contained only bacteria of a filamentous-type, some of which appeared segmented in structure.

Aggregation patterns of plaque cells and those fractionated electrophoretically

The aggregation patterns of unfractionated plaque cells and the more abundant of its various fractions (fractions 29, 30, and 31 in Fig. 6.13) were all found to be similar (Fig. 6.15). The bacterial composition of each fraction was heterogeneous. All showed a single peak at about pH 4.0.

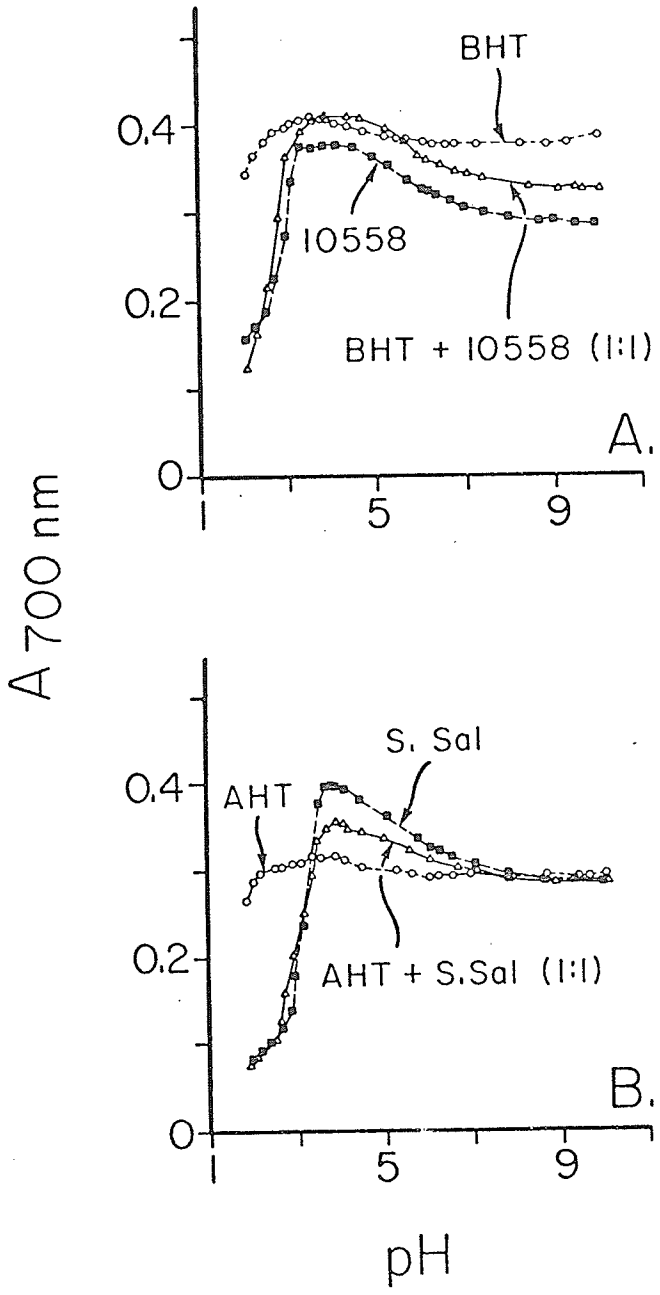


Fig. 6.10. The aggregation patterns of binary mixtures of streptococci and suspensions of the streptococci which make up these mixtures. In each case the bacteria were harvested in the exponential growth phase, washed in distilled water (pH 7.0) and the aggregation medium was distilled water. A. Strep mutans BHT and Strep sanguis 10558 (1:1) B. Strep. mutans AHT and Strep salivarius (1:1).

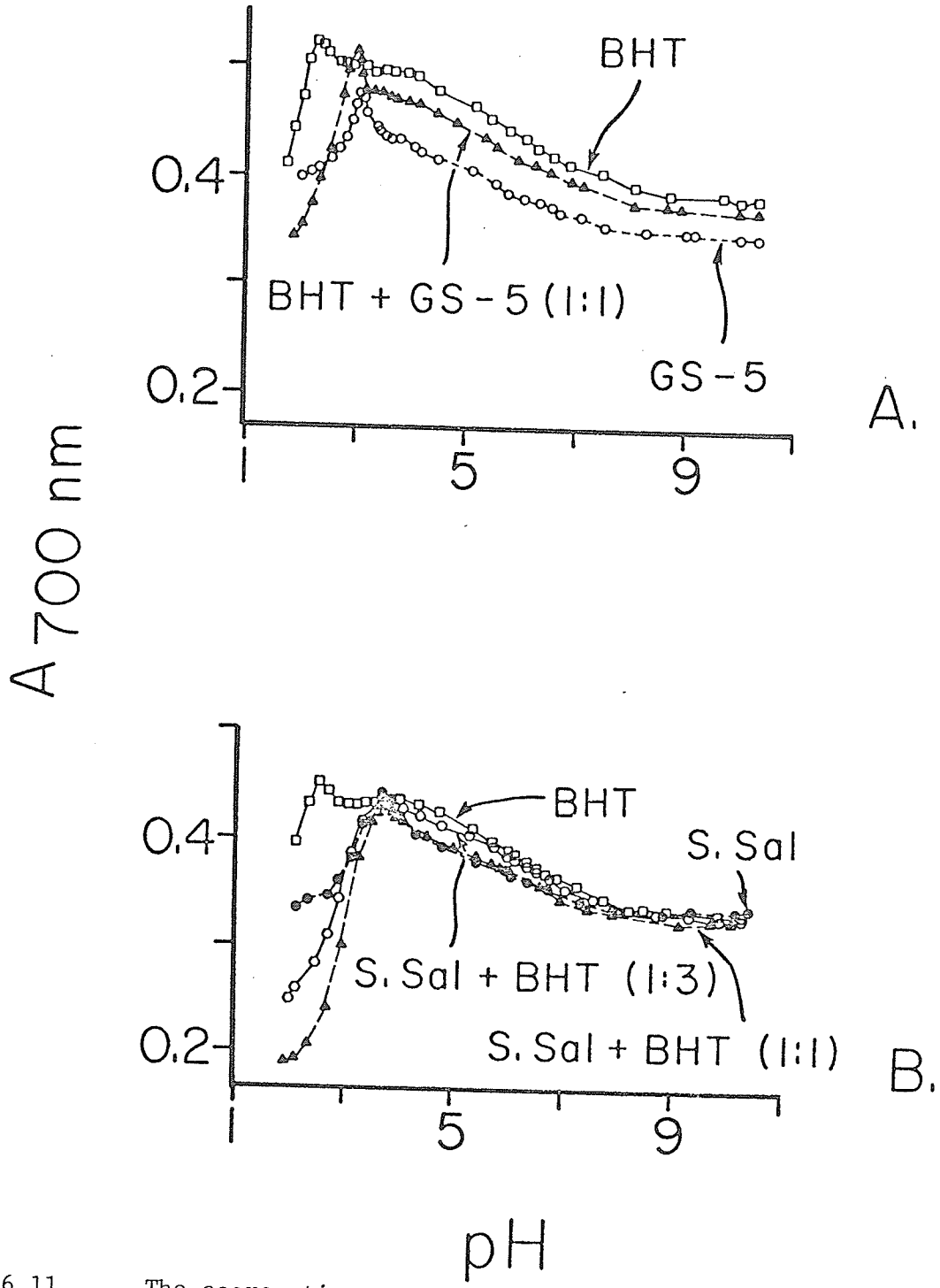


Fig. 6.11. The aggregation patterns of binary mixtures of Streptocci and suspensions of the streptococci which make up these mixtures. In each case the bacteria were harvested in the stationary growth phase, washed with 0.1N NaOH and the aggregation medium was distilled water. A. Strep mutans BHT and Strep mutans GS-5(1:1); B. Strep salivarius and Strep. mutans BHT (1:1 or 1:3).

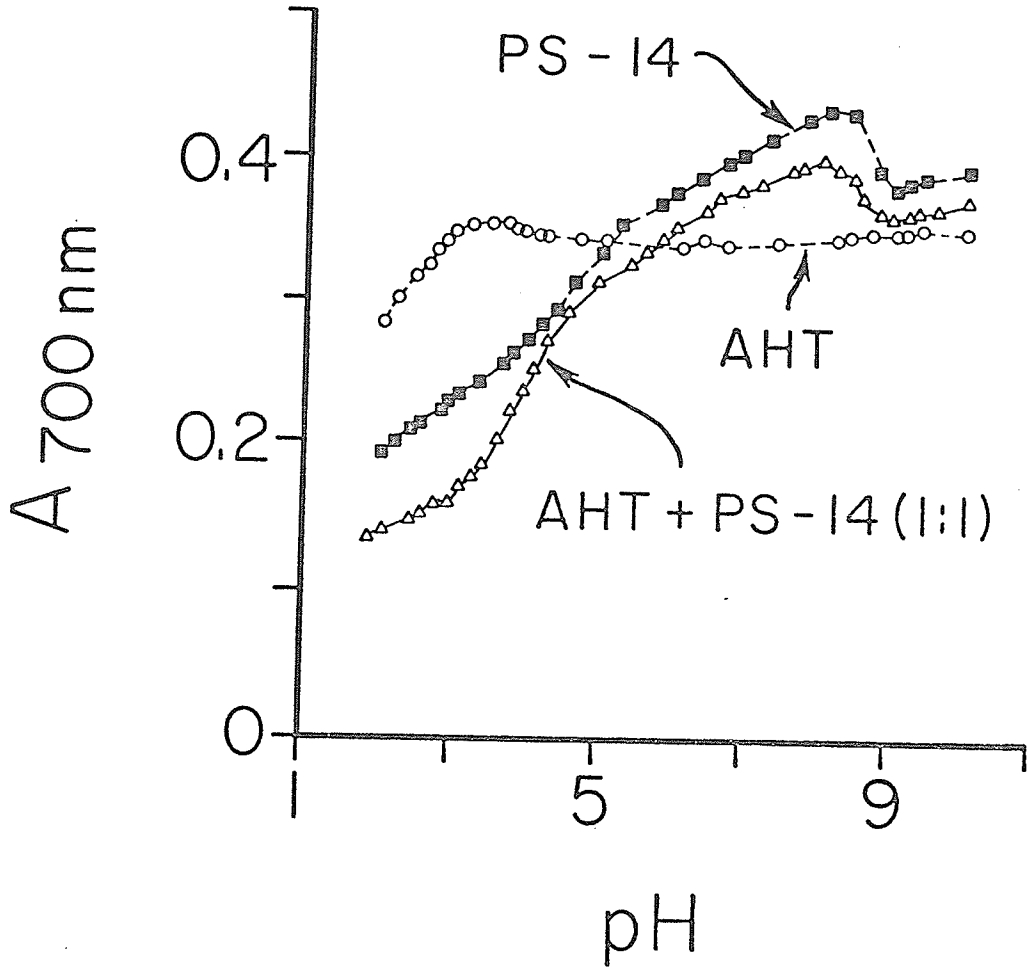


Fig. 6.12. The aggregation patterns of a binary mixture of Strep. mutans AHT and Strep. mutans PS-14 (1:1) and suspensions of each of these streptococci. In each case, the bacteria were harvested in the exponential growth phase, washed with distilled water (pH 7.0) and the aggregation medium was 1 mM $CaCl_2$.

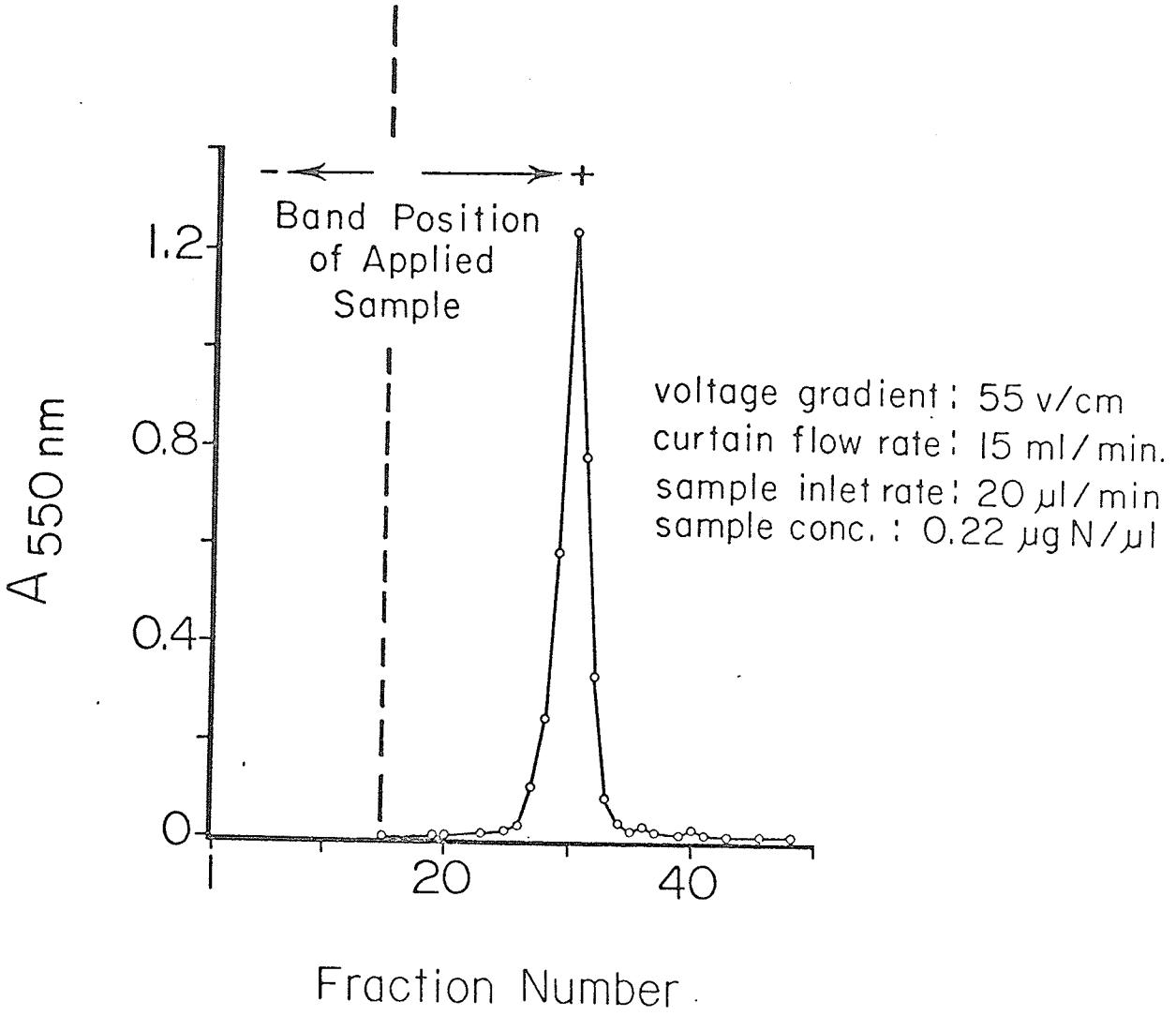


Fig. 6.13. Relative amounts of cells present in fractions of electrophoretically separated plaque cells and the conditions used for continuous particle electrophoresis.

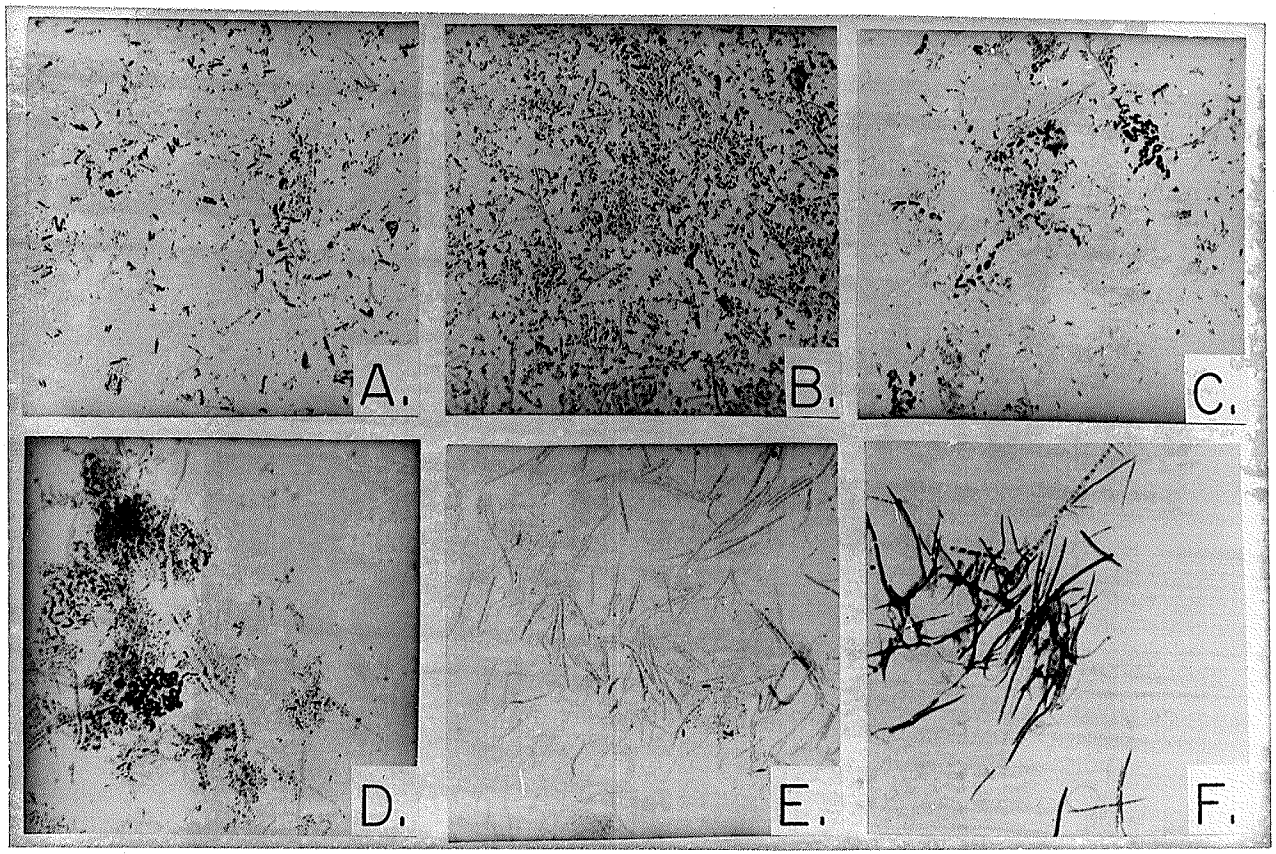


Fig. 6.14. Photomicrographs of the plaque cells present in fractions 19 (A) 27 (B) 30 (C) 36 (D) 43 (E) 47 (F) after continuous particle electrophoresis. (cf. Fig. 6.13). Each fraction was centrifuged (12,000 x g, 30 mins) and the concentrated suspensions smeared on glass slides and stained with crystal violet (original magnification x 1,000).

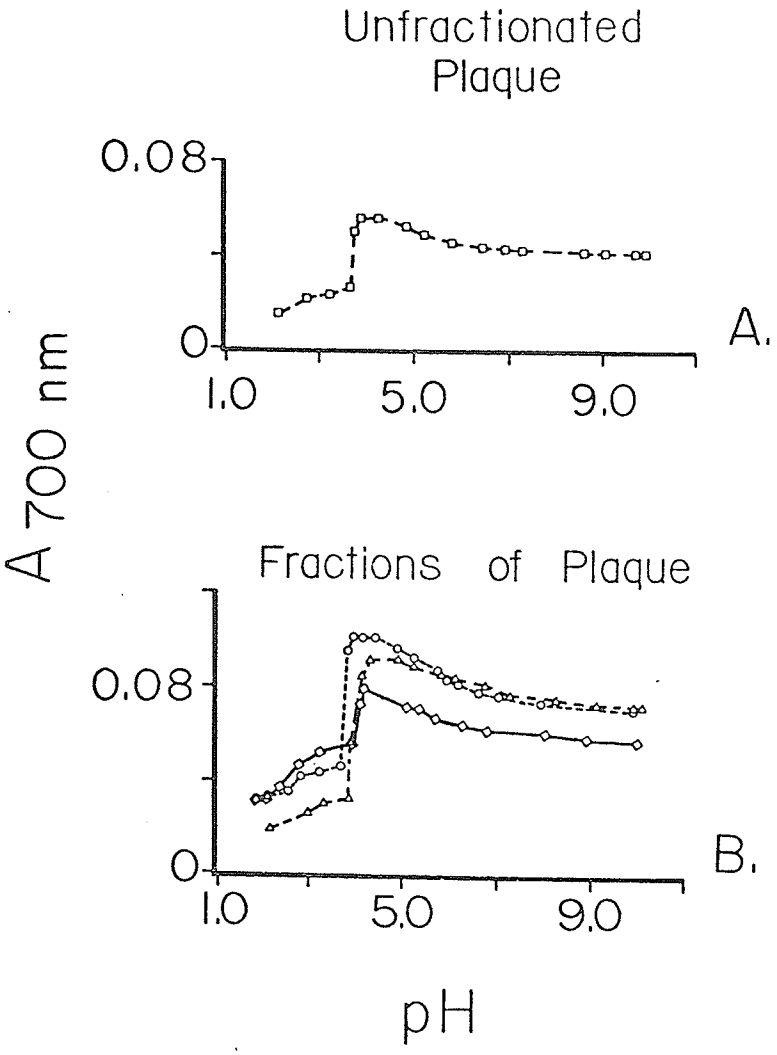


Fig. 6.15. Comparison between the aggregation patterns of unfractionated plaque cells (A) and some fractions of the electrophoretically separated plaque cells (B). The fractions used were 29, 30 and 31 (see Fig. 6.12 and 6.13).

Aggregation patterns of cultured plaque and gingival scrapings

Whether grown anaerobically or aerobically, the bacteria obtained by culturing enamel or gingival scrapings in brain-heart-infusion broth were predominantly gram-positive cocci plus some gram-positive rods. The aggregation patterns for both enamel and gingival scrapings were almost identical and showed only single peaks at about pH 3.0 (Fig. 6.16).

DISCUSSION

Both the electrophoretic and aggregation experiments in the present study have confirmed that the bacteria found in dental plaque are negatively charged at a pH above approximately 4.0 to 5.0 (SILVERMAN and KLEINBERG, 1967 b). The chemical groups most likely to be responsible for most of the negative charge of the bacterial surface are the carboxyl and phosphate moieties (JAMES, 1965). One would expect differences in the number and distribution of such groups to be the basis for the variation in the aggregation patterns observed in the present study with the pure cultures of streptococci (Table VI.1). Whereas the types of surface ionic groups and their proportions are likely to determine the basic pattern of bacterial aggregation, the distribution of the anionic sites may also have an effect since the affinity of divalent cations such as calcium is greater for closely than for widely spaced sites (DIAMOND and WRIGHT, 1969).

Although prior exposure of the streptococci to 0.1 N NaOH or harvesting in either the exponential or stationary growth phase resulted in only slight effects on the aggregation behaviour, sufficient changes occurred with Strep. mutans AHT, BHT, and Ingbritt to suggest that the loss or the uncovering of certain surface components may be

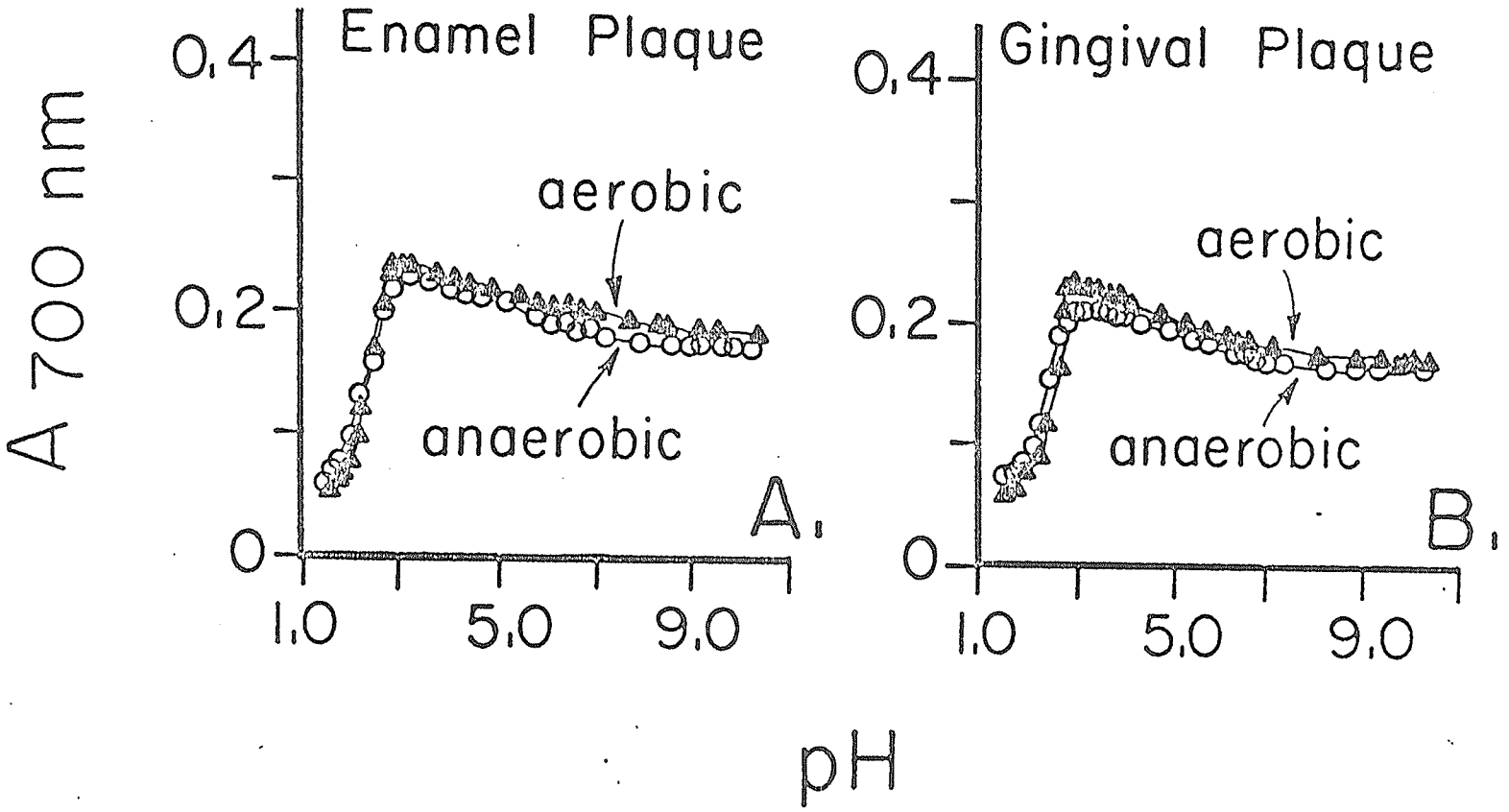


Fig. 6.16. Aggregation patterns of aerobically and anerobically cultured enamel and gingival plaque bacteria.

responsible for the changes in their aggregation behaviour.

The results also show that the aggregation of pure bacterial cultures, as was observed previously with mixed bacteria (SILVERMAN and KLEINBERG, 1967 b), increased when the hydrogen ion concentration, ionic strength or the concentration of divalent cations was increased. Since each of these factors reduces negative surface charge, these results confirm that the ability of these bacteria to remain in suspension is dependent, at least in part, upon surface group ionization (viz. HARRIS, 1951; SILVERMAN and KLEINBERG, 1967 b).

Increasing the ionic strength or adding calcium to the suspending medium raised the pH of maximum aggregation of Strep. mutans strains AHT, BHT, 6715, OMZ 176 and GS-5, Strep. sanguis 10556 and Strep. salivarius only slightly. In contrast, the MA pH of Strep. mutans strains PS-14 and Ingbritt and Strep. sanguis strains 10558 and 10557 was markedly raised by increasing the ionic strength or adding calcium.

At least two factors could account for this difference in effects. Firstly, the ionic strength or calcium levels used may not have been as optimal for those organisms which showed little change in their MA pH as for those which did. Secondly, presence of ionizable surface groups may not be the only factor important in maintaining the suspension stability. For some bacteria, neutralization of the electrical charge alone will not result in marked aggregation since these microbes have a strong affinity for water (NAKAMURA, 1961). For such bacteria, complete aggregation and precipitation from suspension requires not only neutralization of the electrical charge but also removal of the hydration layer. Since polysaccharides are hydrated and have few ionizable groups such substances may constitute important surface com-

ponents for those organisms upon which ionic strength and calcium have a minimal effect. This possibility probably best explains the behaviour of the type III organisms since neither increasing the ionic strength, nor adding calcium nor increasing the hydrogen ion concentration resulted in as marked aggregation as occurred with the other streptococci tested.

While these aspects of the aggregation behaviour of the oral streptococci require further investigation, it is apparent that even for strains of a single species cultured under similar conditions, variability in the surface composition may exist. Such differences could explain the strain variability noted in studies of the adsorption of such streptococci to oral surfaces (Van HOUTE et al., 1970; GIBBONS and Van HOUTE, 1971).

Interbacterial aggregation in binary mixtures

The differences apparent in the aggregation behaviour of single strains of microorganisms was much reduced when combinations of these microorganisms were tested. In the aggregation experiments with binary mixtures of pure cultures of the different streptococcal strains, the pH of maximum aggregation (MA) of the mixture was near that of the microorganism which had the higher MA pH when tested alone. This may be explained as follows. The surfaces of both types of microorganisms will be negatively charged above the higher MA pH. Therefore, little or no aggregation will occur. However, once the pH is lowered below this value, the microorganism with the higher pH of maximum aggregation will be positively charged, whereas the microorganism with the lower MA pH will still be negatively charged. Consequently, the two types of bacteria would aggregate by mutual neutralization of opposite charges (LAMANNA and MALLETTE, 1965). The pH at which this would take place,

the binary MA pH, is also that pH at which one of the microorganisms just acquires a net positive charge. Since this pH is near the MA pH of the microorganism, one can see why its MA pH and the binary MA pH are almost the same (Figs. 6.10, 6.11, and 6.12).

The finding that the binary MA pH was also higher in the presence of added calcium can be attributed to calcium ions substituting for hydrogen ion in reducing the net negative charge of the bacterial surface. This should favour a shift in the pH of maximum aggregation to a higher value.

In both the binary and single organism mixtures, an interesting observation was the apparent irreversibility of the aggregates present when the pH reached the low levels at the end of the titration. Attempts to redisperse some of these aggregates by brief washing with 0.1 N NaOH were only partially successful. However, the organisms that were freed behaved in a similar way as the original suspension when subjected to retitration. Systematic study of this phenomenon amongst the different streptococci was not undertaken and data are not available to ascertain whether the susceptibility of organisms to this effect is a general one or whether it may differ amongst those organisms with differing surface characteristics.

Bacterial aggregation in the formation of dental plaque

The selective colonization of various sites in the human mouth by the oral streptococci has been attributed to differences in their ability to adhere to surfaces (GIBBONS and Van HOUTE, 1973). The results of a study where pure cultures of these bacteria are introduced into the mouths of human subjects seemed to support this hypothesis, since adherence to cleaned teeth and epithelial surfaces appear to be proportional

to the incidence of these organisms in established plaques at these sites (Van HOUTE et al., 1971). In this study it was also shown that the two Strep. sanguis strains tested adhered equally well to the cleaned tooth surface and to preformed plaques. Unless there is some common factor such as calcium involved in the binding in both cases, it is difficult to see how this could occur, since enamel and plaque surfaces are so different.

The present study indicates that interbacterial aggregations are more likely to occur at acidic pH since some bacteria may be below their MA pH and available to facilitate the deposition of the micro-organisms which are not. Because plaque is required for the pH to fall appreciably, this process is more apt to occur during the advanced stages of plaque development and could take two forms. Firstly, acid from the plaques could lower the pH of the saliva (NEUWIRTH and KLOSTERMAN, 1940) and favour interaction amongst the salivary bacteria; secondly, salivary bacteria in close proximity to the plaque could interact with the plaque bacteria.

During the early stages of plaque formation, the bacteria would tend to have a net negative surface charge, since the plaque pH would not have attained a level lower than the MA pH of the bacteria. Under these conditions interbacterial aggregation would be less likely to occur without the aid of calcium and/or calcium linked salivary polymers (SILVERMAN and KLEINBERG, 1967 b).

Analysis of the aggregates formed from whole saliva indicates that they are essentially a calcium phosphate carbohydrate-protein complex. At acidic pH, these aggregates contain less calcium and phosphate and more carbohydrate-protein, whereas at basic pH the ratios are reversed

(KLEINBERG et al., 1971). Some evidence exists suggesting that when subjected to oral environmental conditions the calcium of this complex becomes exposed and able to interact with the anionic sites on the bacterial surfaces. Thus, bacterial entrapment and deposition on the hard and soft oral tissues would be more apt to occur. Consistent with this mechanism is the observation that the removal of calcium with a chelating agent inhibits bacterial aggregation by polymers in saliva and replacement of the calcium restores this capability (GIBBONS and SPINELL, 1969; KASHKET and DONALDSON, 1972).

Depending upon such factors as pH, the presence of calcium, ionic strength, presence of aggregating polymers and the surface characteristics of the bacteria involved, it would be possible by altering one or more of these parameters, to obtain a large number of aggregates of different composition. For this reason, testing the aggregation behaviour of organisms under only a limited set of conditions is unlikely to provide conclusive results.

It has been proposed that dextrans, formed from the metabolism of sucrose by Strep. mutans, play a unique role in attaching these organisms to the enamel surface by a dextran-glucosyl transferase bond (GIBBONS and FITZGERALD, 1969). This hypothesis does not specifically state whether this bond is the one involved in the initial attachment of the microorganisms to the enamel surface or entry into the plaque milieu. Since strains of Strep. mutans examined in the present study were influenced by pH, ionic strength and calcium in a similar manner as were strains of Strep. sanguis and the mixed bacteria obtained directly from plaque (SILVERMAN and KLEINBERG, 1967 b). It is probable that initial attachment of Strep. mutans involves these factors. Assuming this to be so, it becomes necessary to account for the following

observations. Strep. mutans is found most frequently in fissures and carious lesions, less frequently in approximal and least frequently on smooth surfaces (IKEDA and SANDHAM, 1971; KEENE et al., 1972); and it is found in higher numbers in plaques of individuals with greater caries activity (GIBBONS et al., 1973). The simplest explanation is based upon the observation of DRUCKER (1969) that Strep. mutans has a pH optimum for growth lower than many of the other oral streptococci (DRUCKER, 1969). Strep. mutans also favours acid formation at a lower pH than Strep. sanguis (KOMIYAMA and KLEINBERG, 1973). Thus it would appear that rather than being an initiator in human plaque formation Strep. mutans like lactobacilli is an indicator of the acidic condition resulting from increased carbohydrates availability associated with caries activity. Strep. mutans, because of its dextran forming ability, may be an indicator of greater sucrose availability, whereas lactobacilli may reflect the increased availability of other sugars.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Due to the difficulty in obtaining sufficient plaque for *in vitro* investigations and the many technical problems that have to be overcome in order to study plaque *in situ*, many aspects of plaque metabolism and formation are difficult to investigate. For this reason, KLEINBERG (1967a) has developed the suspended salivary sediment (SSS) system, an *in vitro* model which allows one to formulate the approaches and develop methods necessary for the study of dental plaque.

The first study in this thesis examines whether or not each of a number of factors, shown previously to affect the metabolism and the pH response of the SSS system, has a similar effect on the pH and accordingly the metabolism of the dental plaque microflora. In the first series of experiments, both the suspension and glucose concentrations were varied in plaque and sediment incubation mixtures to establish the cell and the substrate conditions under which the two systems have the closest glycolytic activities and produce similar pH curves. This examination was carried out in the presence and absence of salivary supernatant. The cell concentrations yielding similar pH-time curves were 8.3 and 16.7 percent (V/V), respectively. Using these cell concentrations, the effects on the pH curves of (i) salivary supernatant, (ii) glucose, (iii) urea (iv) D(-) and L (+) lactic acid, (v) fluoride and (vi) a salivary fraction containing pH-rise factor were examined. The buffering capacities of the plaque and salivary

sediment mixtures were also compared to determine what influence buffering had on the pH response. This was done both in the presence and absence of salivary supernatant.

In each case, the pH responses were quite similar, especially in the presence of supernatant. However, the plaque suspensions showed a slightly greater ureolytic activity both in the presence and absence of supernatant and a slightly greater buffering capacity only in the absence of supernatant.

The results of these experiments added considerable support to the hypothesis that the acid-base metabolisms of the plaque and sediment microfloras were similar and substantiated the use of the SSS system as a model for studying the metabolism of dental plaque.

The second study in this thesis attempted to test the hypothesis that ammonia formation from salivary urea is responsible for the regional differences in pH observed previously in fasted plaques. The ammonia and urea levels in plaques on the labial and interproximal surfaces of maxillary and mandibular incisors were determined and then related to the corresponding pH. The ammonia levels showed the same pattern as the pH, the maxillary labial plaques having the lowest values, mandibular interproximal plaques the highest and the maxillary interproximal and mandibular labial plaques, intermediate values. On the other hand, plaque urea levels, which were much lower than plaque ammonia levels, were similar at all sites. Titration experiments showed that the buffering capacities of the different plaques were similar and that the difference in pH between maxillary and mandibular incisor plaques could be attributed to their difference in ammonia

concentration. It was concluded that the greater urea availability to mandibular incisor plaques (because of the greater salivary access) would favour a greater number of ureolytic organisms. This would lead to greater ureolytic activity, more rapid formation of ammonia and a higher pH. However, there would be no difference in plaque urea levels, since the greater ureolytic activity would compensate for the higher urea availability at this site. The possible importance of ammonia formation from salivary urea was discussed.

In the third study, certain aspects of the acid or base production diffusion theories, proposed to account for the pH changes and accumulation of acid or base in plaque following exposure to sugar or urea, were examined. After exposure to a urea rinse, the uptake and clearance of urea from both plaque and saliva and the formation and clearance of ammonia from the plaque were monitored. In similar experiments, the uptake and clearance of glucose from plaque and saliva were determined.

The uptake of both urea and glucose by the plaque was very rapid, and the clearance of each from the plaque decreased exponentially. Both the entry and clearance processes were faster for urea than for glucose. On the other hand, the glucose disappearance from saliva was faster than that of urea. During the urea rinse, ammonia accumulated in the plaque at the same rate as the urea was cleared, indicating that urea disappearance from the plaque occurs mainly by bacterial degradation to ammonia. The ammonia disappearance from the plaque was much slower than ammonia formation confirming that the rapid pH changes in the plaque arise from the large difference in the rate constants for these

two processes. By calculation it appeared that only 16 - 26 per cent of the ammonia that could have been formed was actually produced. This agrees with previous findings with salivary sediment and suggests that storage of urea-N has taken place.

In the fourth study, the composition of the free amino acid pool in plaques from different sites on the incisor teeth was established. Analysis of the free amino acid pools of plaques from maxillary and mandibular incisors showed that glutamic acid constituted at least 50 per cent of the total pool. In decreasing order of prominence the pool consisted of glutamic and aspartic acids, proline, ornithine, alanine, lysine, glycine, threonine and serine. Levels of each of these amino acids in the plaques from the various sites were approximately the same, except that glutamic and aspartic acids tended to be higher in mandibular interproximal plaques. The composition of these pools was different from that of hydrolysates of either the plaque bacteria or the plaque matrix.

In a second set of experiments, plaques were sampled before and after rinsing with urea and/or glucose and their amino acid pools examined. Following the urea rinse, aspartic acid and to a lesser extent glutamic acid and alanine showed distinct increases. On the other hand, after the glucose rinse, aspartic and glutamic acids showed large decreases; however, alanine still showed an increase. With the glucose plus urea rinse, glutamic acid rose and fell, aspartic acid decreased slightly and alanine increased significantly.

From this study it was concluded that (i) dental plaque <u>in situ</u> contains a characteristic intracellular pool of free amino acids that

changes in composition when exposed to urea and glucose; (ii) the pools in plaques from different dentition sites generally have similar compositions; (iii) glucose and urea appear to be degraded in the same way as in salivary sediment and form large amounts of alanine when both substrates are present; and (iv) the large amounts of glutamic and aspartic acids and their sensitivity to change in the presence of urea and glucose indicates that these amino acids are important in the amino acid metabolism of dental plaque.

Since plaque formation is still not clearly understood, the final study in this thesis examines several aspects of bacterial aggregation related to plaque formation. In one series of experiments the effect of several physico-chemical factors on the aggregation patterns of pure cultures of oral streptococci were determined. The species of oral streptococci tested were Strep. salivarius (1 strain), Strep. mutans (7 strains) and Strep. sanguis (3 strains).

The difference in the patterns of aggregation of suspensions of these microorganisms in titration experiments (pH 10.0 to 2.0) allowed grouping of the bacteria into three different categories (Type I, II, III). The pH at which maximum aggregation occurred (MA pH) tended to be highest for the Type I organisms, less for the Type II and least for the Type III, though some overlap was apparent. Increasing the ionic strength or adding calcium to the suspending medium resulted in a shift of the MA pH to higher values. This effect was smallest for Strep. mutans strains, AHT, BHT, 6715, OMZ 176 and GS-5, Strep. sanguis 10556 and Strep. salivarius. In contrast, ionic strength and calcium had a large effect on Strep. mutans strains PS-14 and Ingbritt and Strep. sanguis

strains 10558 and 10557.

It was concluded that the differences in aggregation behaviour amongst these oral streptococci reflect differences in the nature of their surfaces. Such differences could be the type and distribution of ionizable groups and/or the presence of hydrated surface components such as polysaccharides.

In continuous particle electrophoresis experiments a partial separation of bacterial morphological types was achieved indicating that differences in the surface charge density of the bacteria naturally occurring in plaques also exists.

When the aggregation of binary combinations of pure cultures of the streptococci was examined it was found that the pattern of aggregation was most like the organisms within the mixture having the higher MA pH. Mixtures containing plaque microorganisms either cultured from the tooth and the gingival margines or obtained directly from plaque behaved in a manner similar to the binary mixtures.

The results of this study support the hypothesis that an electrostatic mechanism is involved in bacterial aggregation phenomenon and has clarified some of the factors which may be involved in plaque formation.

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