STUDIES ON THE RELEASE OF CALCIUM AND PHOSPHORUS FROM DENTAL PLAQUE AND SALIVARY SEDIMENT

A Thesis

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by

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CHAPTER I

INTRODUCTION

In addition to comprising the major elements of the mineral portion of the hard tissues of the body, calcium and phosphorus are involved in many important biological phenomena. The factors affecting the solubility of these elements have been extensively studied since such factors would have a major influence upon calcium and phosphorus availability to cells and tissues. One such factor most effective in the oral cavity is pH.

Bacterial deposits that accumulate on the surfaces of teeth, termed dental plaque, have been shown to play a primary role in the initiation of both dental caries and periodontal disease. These deposits contain high levels of calcium and phosphorus largely in the form of calcium phosphate salts. Since plaque metabolism results in rapid alterations in the pH this would be an important determinant not only of enamel dissolution and calculus formation but also of the efficacy of therapeutic agents to combat both conditions. Fluoride, which is presently widely used to prevent dental caries, has been shown to inhibit acid production during fermentation of dietary carbohydrate by oral microorganisms and reduce the solubility of tooth minerals.

The present study attempts to clarify the complex interrelationships between the acid-base metabolism, fluoride and calcium phosphate solubilization in dental plaque.

Since sufficient quantities of plaque are not usually available for detailed chemical analysis and since plaque in situ studies are technically difficult, salivary sediment as well as plaque was used in this study. Under conditions of controlled cell concentration the acid-base metabolism of both salivary sediment and plaque are similar (Kleinberg, 1970). However, plaque contains much higher levels of calcium and phosphate than does sediment. Therefore, experiments were also carried out in which hydroxyapatite and dicalciumphosphate (the two salts shown to be present in dental plaque, the latter presumeably as brushite, Kaufman and Kleinberg, 1973) were added.

This thesis has examined (1) the release of calcium and phosphate from both dental plaque and salivary sediment during incubation with glucose and urea, (2) the effect of pH at each of several levels between 4.0 and 9.0 on the release in both the presence and absence of dicalciumphosphate or hydroxyapatite, (3) the effect of fluoride and of various acids (typically those produced by the oral bacteria during carbohydrate metabolism) on the release.

Chapter II consists of a review of the literature pertinent to the acid-base metabolism and calcium phosphate solubility

phenomena on dental plaque. The experimental portions are reported in Chapters III and IV. Chapter III deals with the effect of pH on the calcium and phosphate released from dental plaque and salivary sediment; Chapter IV deals with the effect of fluoride on this release. In Chapter V the implications of the study to dental caries and periodontal disease are considered. Chapter VI contains the summary and conclusions of the thesis.

CHAPTER II

REVIEW OF THE LITERATURE

Dental Plaque

The bacterial deposits which accumulate on the surfaces of the teeth have been studied since the beginning of the century for their role in dental caries and inflammatory periodontal disease. These deposits, called dental plaque, consist of bacteria, intercellular material (mostly protein and polysaccharide) and fluid (saliva and, in many cases, fluid from the gingival crevice; Kleinberg, 1970). Plaque is usually not found in those regions of the dentition normally abraded by the oral musculature or by coarse dietary constituents. However, the pits and fissures, and the approximal surfaces and regions of the teeth adjacent to the free margin of the gingivae are the most susceptible and common areas of plaque accumulation.

Acid-Base Metabolism of the Plaque

Immediately following the ingestion of fermentable carbohydrate the microorganisms of the dental plaque rapidly form acid (Stephan, 1940; Stralfors, 1950; Kleinberg, 1961). In experiments with glucose or sucrose rinses, the pH of the dental plaque shows a rapid fall, reaches a minimum which persists for a few minutes and then slowly rises until the

pre-rinse level is reached between 30 minutes and several hours thereafter (Stephan, 1940; Kleinberg and Jenkins, 1964). Most significant is that the acid accumulation, if sufficient, may lead to the dissolution of the underlying dental enamel and initiation and progression of a carious lesion (Stephan, 1944; Stralfors, 1950).

The plaque bacteria are also capable of rapid base formation when urea is substituted as substrate (Stephan, 1943; Frostell, 1959; 1960; Singer and Kleinberg, 1969). Rapid formation of ammonia results in a sharp rise in the pH; however, once the urea has been utilized the pH slowly falls to the starting level. The low solubility of calcium phosphate at high pH (Fosdick and Starke, 1939) is conducive to the accumulation of calcium and phosphate within the plaque. When such a process occurs, the deposit is called dental calculus.

Salivary Sediment as a Model for Plaque Studies

Sufficient plaque for most metabolic studies requiring aliquots for analysis is difficult to obtain. Further, difficulties of methodology of *in situ* studies of human plaque necessitated the development of an *in vitro* model in which such *in vivo* changes as the pH curves seen with glucose and urea could by simulated (Kleinberg, 1967). The model consists of salivary sediment suspended in the supernatant of centrifuged wax-

stimulated whole saliva and has been named the suspended salivary sediment (SSS) system (Kleinberg, 1967). The experiments leading to the development of the model showed that high cell concentrations must be present in order to produce the pH curves seen in plaque in situ. Studies with pure cultures of plaque bacteria in buffers have shown a similar need for high cell concentrations (Stephan and Hemmens, 1947; Stralfors, 1950).

Some differences in the bacterial composition of salivary sediment and dental plaque have been reported (Krasse, 1954; Gibbons et al., 1964). However, experiments carried out comparing the acid-base metabolisms showed little or no difference. Salivary sediment contains more mammalian cells (mainly epithelial which are metabolically inactive, Molan and Hartles, 1967) than plaque and as a result a lower bacterial cell concentration. This difference has a greater effect upon metabolism than any differences in the incidence of specific bacterial species in the two bacterial systems (Singer and Kleinberg, 1970).

A number of studies have revealed differences between the calcium and phosphate levels of plaque and sediment. Harucki and Moore (1957) have shown that sediment can concentrate calcium when exposed to solutions containing up to four times the level of saliva. Dawes and Jenkins (1962) estimated that plaque con-

tains nineteen times as much calcium as does saliva while sediment has only three times as much. The phosphate content of plaque was twice that of sediment. These observations suggest that addition of calcium phosphates to salivary sediment may be required in studies where inhibitors of both acid formation and solubilization of calcium phosphates (eg. fluorides and phosphates) are being examined.

Depending upon the relative availability of carbohydrate and nitrogenous substrates, the pH of plaque *in situ* or in salivary sediment mixtures *in vitro* can vary between a low of apporximately 4.0 and a high of 9.5 (Kleinberg, 1961; 1967). Such shifts of pH at the plaque-enamel interface could alter the solubility of calcium phosphate drastically and would certainly be an important determinant of the changes in calcium phosphate levels at this interface and within the plaque itself.

Calcium and Phosphate Levels in Plaque

Not only are the calcium and phosphate levels severalfold higher in plaque than in salivary sediment, but these levels in plaque vary with the age of the plaque and with its location in the oral cavity (Dawes and Jenkins, 1962; Kaminsky and Kleinberg, 1967).

The calcium and phosphate levels of plaque are high on the first day, fall markedly on the second and rise slightly or

remain unchanged on the fourth day (Kleinberg et al., 1971). Recent x-ray diffraction studies have shown that the predominant forms of calcium phosphate observed in dental plaque were brushite and apatite (Kaufman and Kleinberg, 1973). Further, amorphous and poorly crystalline forms occur more frequently in early plaques, in the plaque of young subjects and in areas of the mouth where plaques are usually at a more acidic pH. Crystalline forms of calcium phosphate (brushite and apatite) are more frequent in the plaques of older subjects, in plaques which themselves are older and in plaques from areas of higher pH. This would suggest that calcium phosphate in early plaque undergoes amorphous to crystalline transition.

Plaques located on the mandibular incisors show higher calcium and phosphate levels than plaques on the surfaces of the maxillary incisors (Kaminsky and Kleinberg, 1967). In a study of adolescent and adult subjects the calcium and phosphate levels of similar plaques (i.e. same age, same dentition location) were higher in the younger subjects (Kleinberg et al., 1971).

Calcium Phosphate Solubility

Both its wide distribution and its apparent importance in biological phenomena had lead to extensive study of calcium phosphate to determine the factors controlling its solubility. Levinskas and Neuman (1955) found that the solubility of calcium

phosphate increases with an increase in the amount of solid added to a given volume of solution. Logan and Taylor (1935) observed the exact opposite. As a result of studies such as these, hydroxyapatite was considered by some investigators not to have a solubility product constant.

The main reason that calcium phosphate shows incongruent solubility is that it may exist as several forms and may easily change from one form to another in aqueous media. For example, in an acidic medium, hydroxyapatite dissolves and forms salts of lower Ca/P ratio, such as secondary calcium phosphate ($CaHPO_4$) (Gray et al., 1962). If the medium is neutral or alkaline, hydrolysis of hydroxyapatite occurs and a surface complex of alkaline calcium phosphate ($Ca_2HPO_4(OH)_2$) forms (Rootare et al., 1962). Thus, the calcium and phosphate in equilibrium with the solid may not only involve the solubility of a particular calcium phosphate but also a transformation during the period of equilibration. Since the rate and extent of transformation will depend upon the surface area of the solid, the equilibrium will depend upon the solid to solution ratio and on particle size.

In addition to pH and solid to solution ratio, several other factors, in particular the calcium and phosphate content of the fluid, its ionic strength, the presence of certain ions such as bicarbonate, influence the solubility of hydroxyapatite.

Examination of the solubility of calcium phosphates by a large variety of organic acids (Johnston, 1952; Koulourides and Buonocore, 1961) has demonstrated that in addition to the concentration of hydrogen ion, the structure of the organic molecule is an important contributing factor. As well as the strength of an organic acid (appraised in terms of its dissociation constant), Koulourides and Buonocore (1961) found that the spatial arrangement of functional groups and the existence of multiple charges were important factors determining the solubility potential of an organic acid. Thus, acid decalcification at the plaque-enamel interface would depend both on the pH and on the types of acids present.

The major acids formed during the catabolism of carbohydrate by the plaque bacteria are acetic, propionic and lactic acids (Muntz, 1943: Gilmour and Poole, 1967). Sandham and Kleinberg (1970) have shown that the same acids are formed in the SSS system during glucose breakdown.

Calcium and Phosphate of Plaque as Reservoirs for Protection of Enamel Dissolution and Facilitation of Enamel Remineralization

When calcium and phosphate ions are added to acidic buffers, such a mixture retards the attack of enamel by acid (Hills and Sullivan, 1958; Stralfors, 1959). Even at a pH as low as 3.5, Besic (1953) failed to produce enamel destruction in an acidic

buffer previously saturated with calcium and phosphate by equilibration with excess tricalcium phosphate. One, therefore, would expect localized demineralization of the enamel to occur when the fluid within the dental plaque becomes unsaturated with respect to hydroxyapatite as can occur when bacterial degradation of fermentable carbohydrates causes a large fall in plaque pH. Under these conditions, the calcium and phosphate reservoirs of the plaque may only protect the enamel from acid attack if sufficient amounts of the bound calcium and phosphate are solubilized. Assuming that some of the calcium and phosphate of the enamel is solubilized, should the pH subsequently rise, the calcium and phosphate in solution should favour precipitation and enamel remineralization.

Animal experiments have demonstrated that readily soluble phosphate has a cariostatic influence (Harris et al., 1965). In a recent three year study on 1500 children, calcium sucrose phosphate incorporated into food significantly reduced dental caries between 20 and 35% (Smythe, 1971). A chewing gum containing dicalciumphosphate by increasing the calcium and phosphate levels of the dental plaque (Chatterjee and Kleinberg, unpublished results) appears to reduce dental caries in children (Finn and Jamison, 1967).

Koulourides and Reed (1964) found that fluoride, calcium

and phosphate synergistically protect the enamel from softening in an artificial bacterial plaque-tooth system. In addition to favouring remineralization of the enamel, phosphates may buffer the plaque and minimize the harmful effects on the enamel of the acids produced in the plaque.

Inhibitors of Calcification

Inorganic pyrophosphate has been shown to inhibit both calcification in an in vitro system of physiologic solutions (Fleisch and Bisaz, 1962) and the caries process in rodents (Nizel and Harris, 1960). Further, inorganic pyrophosphate reduces the acid solubility of hydroxyapatite and inhibits its nucleating ability in a metastable calcifying solution (Amdur et al., 1963). Of significance is the clinically related finding of Vogel and Amdur (1967) which shows higher orthophosphate and lower pyrophosphate levels in the parotid salivas of rapid calculus formers than in the salivas of slow calculus formers. Fleisch, Bisaz and Care (1964) reported that an increased urinary pyrophosphate results when the diet is supplemented with orthophosphate. If the salivary pyrophosphate level is also increased, then orthophosphate supplements might lead to decreased calculus formation.

Another biological inhibitor of decalcification is phytate, the hexaphosphate of myo-inositol and a substance removed from sugars and cereals during industrial refinement. It may function by adsorbing to enamel and reducing the solubility of the

hydroxyapatite (Jenkins et al., 1959). Kaufman and Kleinberg (1970) have shown that adsorption of the phytate molecule to an anion exchange resin is inversely related to the pH. Phytate may also be capable of preventing the transformation of amorphous calcium phosphate to crystalline forms within plaque (Kleinberg et al., 1971).

Recent studies have shown that some diphosphonates inhibit the crystallization of hydroxyapatite in vitro and further that its administration orally prevents calcification of the kidney and aorta of the rat (Francis, 1969; Fleisch et al., 1969). As a result of such experiments, it has been suggested that diphosphonates be used in man for the treatment of diseases that involve the deposition of calcium in soft tissues and dental calculus.

Calcium and Phosphate in the Regulation of Physiological Processes

In addition to providing the major elements of the mineral portion of the hard tissues of the body, calcium and phosphorus are also involved in many important biological phenomena.

Vasington and Murphy (1961, 1962) have shown that isolated mitochondria can accumulate large amounts of calcium during electron transport which may be accompanied by phosphate and H^+ ejection (Lehninger et al., 1963; Saris, 1963; Engstrom and De Luca, 1964). Electron microscopy has revealed massively loaded calcium phosphate granules in mitochondria (Lehninger

et al., 1963). Similar granules have been observed *in situ* in a variety of tissues (osteoclasts, chondrocytes, kidney) and lower organisms (protozoa). Present evidence indicates that these calcium phosphate deposits are normally of an amorphous nature (Greenwalt et al., 1964) and are stabilized intracellularly into micropackages and then transported to extracellular calcification sites (Lehninger 1970).

An important aspect of calcium metabolism in mammalian cells is the fact that the extracellular calcium ion concentration is normally 100 to 10,000 times higher than the intracellular calcium ion concentration. Much of the intracellular calcium is unionized, and it is unequally distributed between the various cell compartments. Nevertheless, calcium exchanges rapidly between cell fluids and subcellular compartments. The concentration of phosphate in the medium influences the rate of uptake of calcium and the distribution of calcium within various cell compartments (Rasmussen, 1971). Moreover, membrane transport of phosphate is a calcium dependent process.

Although the mechanisms involved are not fully understood, calcium appears to be involved in the excitation-contraction coupling of muscle. Its release is associated with the muscle sarcoplasmic reticulum and is dependent upon pH (Nakamaru and

Schwartz, 1970; Conway, 1957). Change in pH not only affects calcium binding to or release from membrane sites but also affects the release of an acidic protein from muscle sarcoplasmic reticulum capable of binding considerable quantities of calcium (Stephens, 1969; MacLennan & Wong, 1971).

Calcium is released from blood platelets when incubated with thrombin, latex particles or sodium fluoride (Murer and Holme, 1970). In blood, the released calcium may participate in such processes as clotting and calcium homeostasis.

Calcium may play a role in the adhesion of cells. The calcium may (a) stabilize the arrangement of the intercellular macromolecules linking cells, (b) form a link between negatively charged surfaces of adjacent cells and, (c) reduce the negative surface charge and allow adjacent cells to aggregate.

Thus, factors that affect the availability of calcium and phosphate would indirectly have important regulatory effects on the above processes.

Calcium and Phosphate in Plaque Formation

Dawes (1964a and 1964) observed that the addition of calcium ions to submandibular saliva leads to the precipitation of protein which may contribute to the formation of the dental plaque matrix. McGaughey and Stowell (1966) examined the adhesion of salivary mucin to glass and hydroxyapatite powder.

Both calcium and phosphate were necessary for adhesion of the mucin to glass, but phosphate inhibited the attachment to hydroxyapatite. They suggested that both calcium and phosphate were required for mucin attachment and that enamel phosphates were the sites where the calcium of the mucin molecule became attached.

Hydroxyapatite powder exposed to mixed saliva adsorbs salivary proteins more rapidly at an acidic than at a neutral pH (Hay, 1967). Since preferential solubilization of the calcium of hydroxyapatite occurs at an acidic pH, (Gray and Francis, 1963) more phosphate sites will be exposed and may enable the calcium-mucin complex to attach. The pH also affects the charge on calcium phosphate (Somasundaran, 1968). At an acidic pH, calcium phosphate becomes more negatively charged and thus should attract the calcium-mucin complex.

Varying the pH of saliva by titrating with acid or base (Kleinberg et al., 1971) showed two aggregation maxima, an acidic one -- where the aggregate contained small amounts of calcium and phosphate and large amounts of carbohydrate-protein, and a basic one -- where the contents of calcium and phosphate and carbohydrate-protein in the aggregate were reversed. The release of bound calcium and evidence for denaturation led these investigators to propose that following secretion,

the salivary mucin undergoes configurational change, exposing the calcium and preparing the calcium-mucin complex for adherence to phosphate sites on the enamel surface.

The studies of Silverman and Kleinberg (1967) showed that calcium favoured the aggregation of plaque microorganisms, and this would occur with or without plaque or salivary protein present. Without protein, calcium would simply neutralize the negative charge on cell membranes. With protein, the calcium would also favour bridging of protein molecules between adjacent cells and favour the formation of a floc.

Fluoride in Plaque

The concentration of fluoride in dental plaque is high; Hardwick and Leach (1962) reported figures between 6 and 180 ppm. The fluoride content is influenced by the fluoride levels of drinking water -- concentration in the plaques of children from a town with no fluoride in the water averaged 25 ppm compared with 47 ppm in a town with 2 ppm fluoride in the water (Dawes et al., 1965). Fluoride has a great affinity for calcium phosphate and accordingly is incorporated in high concentration into bone and tooth mineral. Although most of the plaque fluoride is in bound form (Jenkins, Edgar, Ferguson, 1969), the greater part does not appear to be associated with the plaque matrix but appears to be present within the bacteria.

Fluoride Inhibition of Acid Formation in Plaque

Wright and Jenkins (1954) demonstrated that fluoride at levels as low as 0.5 ppm could inhibit the fall in pH in wax-stimulated saliva incubated with glucose. Fluoride inhibition of acid formation has also been demonstrated in salivary sediment (Sandham and Kleinberg, 1969) and in pure cultures of streptococci (Weiss et al., 1965; Hamilton, 1969a and b) and shown to be more effective at acidic pH (Jenkins, 1959).

Inhibition by fluoride of glucose utilization and formation of storage polysaccharide in mixed and pure cultures have also been demonstrated and shown to be more effective at acidic pH. The results of these studies indicated that fluoride acted in or in association with cell membranes, its primary effect being that of inhibiting glucose uptake. Uptake of lactic acid is also inhibited by fluoride and the present study shows that fluoride also inhibits utilization of urea.

Lilienthal (1956), however, found that fluoride had no effect on acid production in salivary sediment incubated with glucose or sucrose. Jenkins (1959) pointed out that absence of inhibition is most likely due to the Lilienthal experiments being carried out at neutral pH where fluoride does not inhibit acid production unless present at high levels. Bramstedt et al., (1957) found that at pH 6.8, 2.2 ppm fluoride stimulated the pH

fall when either streptococcus salivarius or lactobacilli casei were incubated with glucose. Sandham and Kleinberg (1969) also demonstrated that fluoride stimulated the decrease in pH in salivary sediment mixtures incubated with glucose, if the pH fall was not below approximately 5.5.

Jenkins (1959) has shown that fluoride in the presence of calcium, magnesium and phosphate stimulated the pH fall of saliva-glucose mixtures slightly.

Kanapka et al., (1971) demonstrated that 2.4 mM fluoride inhibited glucose uptake, glycogen synthesis, glucose-6-phosphate and ATP formation in streptococcus salivarius. These results are consistent with the hypothesis that fluoride in some way interacts with the sugar transport system; they believe it does this by inhibiting the activity of enolase and as a result the supply of phospho-enolpyruvate for phosphorylation of membrane carrier.

The experiments of Lehninger, Rossi and Greewalt (1963) have indicated that phosphate, and calcium form hydroxyapatite in or in association with the mitochondrial membrane. If the same phenomenon occurred at the bacterial cell membrane then the presence of fluoride might lead to the formation of fluorapatite which is less soluble than hydroxyapatite. This in turn might lead to less phosphate being available to the cell,

perhaps resulting in a decrease in the rate of acid formation.

Fluoride Effects on Calcium Phosphate

At low and high concentrations fluoride reacts with dental enamel and hydroxyapatite differently (Leach, 1959). At low concentration, fluoride exchanges for OH^- to form fluorapatite according to the following equation:

$$Ca_{10}(PO_4)_6(OH)_2 + 2F^- \longrightarrow Ca_{10}(PO_4)_6F_2 + 2\ OH^-$$

No exchange with phosphate occurs since exchangeable phosphate does not vary during the uptake of fluoride (Falkenheim and Hodge, 1947; Neuman and Neuman, 1958; Neuman et al., 1950). At high fluoride concentrations, however, double salt decomposition takes place according to the following reaction (Leach, 1959; McCann, 1953; McCann and Bullock, 1955):

$$Ca_{10}(PO_4)_6(OH)_2 + 20\ F^- \longrightarrow 10\ CaF_2 + 6PO_4^{\equiv} + 2\ OH^-$$

Thus, the gross reaction involves breakdown of the hydroxyapatite crystal of the enamel to form calcium fluoride and at the same time liberation of phosphate, carbonate and other soluble ions of the lattice surface. According to Gray et al., (1962), the surface coating of CaF_2 protects the hydroxyapatite crystals against further dissolution. McCann (1968) showed that calcium fluoride is soluble in saliva to the extent of 12-15 mg/l; whereas, fluorapatite is essentially insoluble. He concluded that calcium fluoride upon dissolution would provide fluoride for the formation of fluor-apatite. Further, caries reduction by fluoride would depend

upon the stability of fluorapatite in contact with the salivary fluids.

Fluoride favours the formation of apatite crystals from solutions saturated with calcium and phosphate (West and Storey, 1972). It is this property that is believed to be responsible for the remineralization and rehardening of enamel that occurs in the presence of fluoride. Koulourides (1961) found that 1 ppm fluoride favours rehardening of enamel by a factor of five. This is consistent with the findings of Dowse and Jenkins (1957) that the fluoride content of early carious enamel is two to three times that of intact enamel. Interestingly, fluoride favours formation of apatite crystals from metastable calcifying solutions, when other crystalline species such as brushite or octacalcium phosphate would otherwise form (West and Storey, 1972).

CHAPTER III

EFFECT OF pH ON THE RELEASE OF CALCIUM AND PHOSPHATE FROM DENTAL PLAQUE, SALIVARY SEDIMENT AND CALCIUM PHOSPHATE SUSPENSIONS

INTRODUCTION

Fermentation of dietary carbohydrate by the dental plaque bacteria results in a decrease in the plaque pH and conditions favourable for enamel dissolution and dental caries (Stephan, 1944). On the other hand, base formation during the metabolism of nitrogenous substrates, particularly urea, results in a rise in the plaque pH and conditions favourable for calculus formation (Kleinberg and Jenkins, 1964).

High levels of calcium and phosphate have been found in dental plaque (Dawes and Jenkins, 1964) which x-ray diffraction analysis has shown is present as an amorphous calcium phosphate, brushite and/or apatite (Kaufman and Kleinberg, 1973). The amorphous calcium phosphate was similar to that observed in the carbohydrate-protein material precipitated from saliva under slightly acidic conditions (Kleinberg et al., 1971).

Kleinberg (1970) has postulated that the continuous deposition of this material from saliva maintains a reservoir of calcium and phosphate in the plaque which protects the enamel of the tooth from solubilization when the pH of the plaque becomes acidic.

Further, that basic conditions in the plaque ensure their accumulation and favour their conversion to less soluble calcium phosphates and thus to clinical calculus (Kleinberg, 1970). As a test of this hypothesis, the present study has examined whether or not the calcium and phosphate of the plaque can be mobilized and how this mobilization is influenced by the wide range of pH observed in plaque in situ.

Experiments were carried out with suspensions of plaque, suspensions of salivary sediment, suspensions of either hydroxyapatite or dicalciumphosphate (the two forms of calcium phosphate other than amorphous calcium phosphate that have been observed in dental plaque; Kaufman and Kleinberg, 1973) and suspensions of sediment containing either of these two forms of calcium phosphate.

Salivary sediment contains very low levels of calcium and phosphate (Dawes and Jenkins, 1962) but yields pH changes similar to plaque when incubated with glucose or urea. Thus, the experiments with sediment and those with suspensions of brushite and hydroxyapatite permit a separation of the cellular and calcium phosphate aspects of the phenomenon under study which is not possible with plaque alone.

The effect of pH on calcium and phosphate release during several hours of incubation of the above systems at 37°C was studied in the following three types of experiments. Firstly,

the pH was changed metabolically by incubating plaque and sediment suspensions with either glucose or urea. This produced rapid and large changes in pH during the incubation similar to those seen in dental plaque *in situ* (Kleinberg, 1961 and 1967). (With glucose the pH rapidly falls from neutrality and is capable of reaching a pH between 4.0 and 5.0; with urea the pH rapidly rises to about 9.0 to 9.5). Secondly, the pH was held constant between 4.0 and 9.0 during similar incubations of sediment and plaque mixtures from which glucose and urea were omitted and suspensions of hydroxyapatite and dicalciumphosphate from which sediment and plaque were omitted. Finally, the major acids formed during the catabolism of glucose (lactic, acetic and propionic; Sandham and Kleinberg, 1970) and HCl were each added to sediment mixtures in a manner which duplicated the pH changes seen when mixtures are incubated with glucose. The purpose of these experiments was to determine whether or not amongst these organic acids and between these acids and HCl there was any major difference in the pattern of calcium phosphate release.

METHODS

Preparation of Plaque and Salivary Sediment Incubation Mixtures

Plaque (200-300 mg wet weight) was collected from 4 or 5 donors who had not cleaned their teeth for 3 days nor eaten within 12 hours of plaque collection which occurred on the fourth

day between 9:00 and 10:00 a.m. The plaque was removed with a stainless steel spatula and immediately dispersed in deionized water (450 ul) contained in a centrifuge tube (Spinco Model 152 microfuge tube) chilled in cracked ice. The plaque suspension was centrifuged (1740g for 20 minutes) and the pellet (approximately 150 ul) was washed twice with 3 volumes of cold distilled water before resuspending at a concentration of 25 per cent (V/V).

Salivary sediment was prepared from wax-stimulated whole saliva collected from subjects who had not cleaned their teeth nor eaten for 12 hours before saliva collection. After centrifuging the saliva (1740g for 20 minutes) the sediment was washed twice with 5 volumes of distilled water and then resuspended at a concentration of 50 per cent (V/V). All preparative procedures were carried out in a cold room at $4^{o}C$.

In the salivary sediment system used in this study salivary supernatant was excluded and deionized water used instead. Plaques in different regions of the oral cavity are exposed to variable amounts of saliva. Since there is evidence suggesting that salivary calcium and phosphate would suppress the solubilization of calcium and phosphate from enamel and presumably from dental plaque (Fosdick and Starke 1939), release of plaque calcium and phosphate was restricted to conditions where saliva was absent. The release of these elements in the presence of saliva

would be the subject of a later investigation. Therefore, plaque and sediment were washed with deionized water in order to remove residual saliva and plaque fluid (Edgar 1972). Both contain calcium and phosphate, the presence of which would be expected to inhibit the release of these substances from plaque or sediment.

Plaque and salivary sediment mixtures (600 ul) were prepared having the following compositions and final concentrations (i) either plaque at 8.3 per cent (V/V) or sediment at 16.7 per cent (V/V) (ii) no substrate, or glucose or urea at either 2.8 or 28 mM and (iii) no calcium phosphate, or 2.0 mg of either hydroxyapatite or dicalciumphosphate.

Plaque and sediment were compared at 8.3 per cent and 16.7 per cent respectively since similar pH changes occur with glucose and urea at these concentrations (Singer and Kleinberg, 1970).

Two forms of calcium phosphate of different calcium to phosphate ratio and solubility were used. Hydroxyapatite with a Ca : P ratio of 1.67 is relatively insoluble whereas dicalcium-phosphate with a Ca : P ratio of 1 is quite soluble. The hydroxyapatite used in this study was prepared as follows: 100 ml M/10 H_3PO_4, 1350 ml saturated $Ca(OH)_2$ and 2550 ml of CO_2-free distilled water were mixed at 37°C and the precipitate formed was allowed to equilibrate for 8 days at the same temperature (Holt, LaMer and Chown, 1925). To prevent bacterial contamination during

the equilibration period a few drops of chloroform were added. Supernatant was then removed by careful suction and discarded. The precipitate was allowed to dry at 37°C, and then broken up in a mortar and pestle and sieved. The mesh size of the powder used in the experiments was 100.

The dicalciumphosphate used in this study was purchased. (Fisher Scientific Company).

Calcium, phosphate and x-ray diffraction analyses on both calcium phosphates confirmed their dicalciumphosphate and hydroxyapatite compositions.

Incubations Procedures

(a) Effect of pH altered by Incubation with Glucose or Urea

Plaque (2) and sediment mixtures (4), 2 of the latter with and 2 without added hydroxyapatite, were incubated with 28 mM glucose or urea for 4 hours at 37°C. At intervals during the incubation the pH was measured with a glass electrode (Beckman 39067) and using a micro-KCl salt bridge and a calomel electrode as the reference (Kleinberg, 1967). At the same time interval, aliquots (15 ul) were removed in duplicate from each mixture, centrifuged (12,400g for 3 minutes at 4°C; Spinco microfuge model 152) and aliquots (10 ul) of the supernatant were analyzed for calcium and for phosphate by micro-adaptations of the glyoxlbis-hydroxyanalin (GBHA) method of Goldstein and Stark-Meyer (1963)

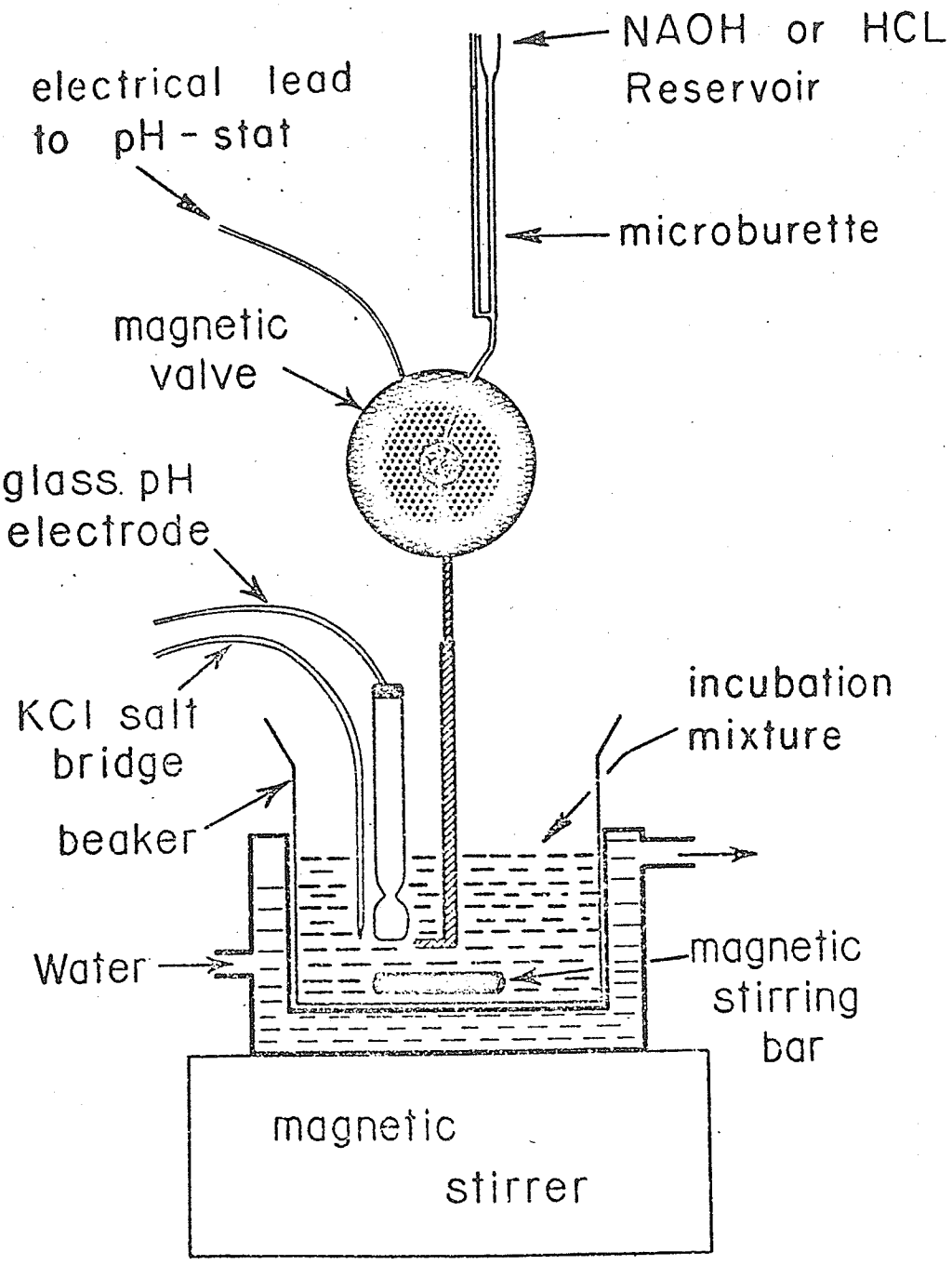


Figure 1. Diagrammatic representation of the pH constant apparatus.

and the phosphomolybdate method of Kuttner and Cohen (1927), respectively. The coefficient of variation of the calcium unknowns was 3.7% and of the phosphate unknowns was 3.2%.

Solubilization of calcium and phosphate from dicalcium-phosphate and from hydroxyapatite was compared in sediment mixtures incubated with 2.8 or 28 mM glucose or urea. The pH attained after the first hour with these substrates changes little during the next 3 hours of incubation; as a result, by the end of 4 hours of incubation one obtains four different levels of pH between about 4 and 9. This type of experiment permitted evaluation of the effect of different pH levels on the solubilization of calcium and phosphate under physiological conditions of incubation.

(b) Effect of pH Held Constant with HCl or NaOH

The release of calcium and phosphate from plaque, sediment and sediment and hydroxyapatite mixtures were examined during 4 hour incubations at pH 4, 5, 6, 7, 8 or 9. The pH was adjusted and held constant by the addition of either HCl or NaOH from a pH-stat (Radiometer Titrator, Type TTT 116 and pH meter, Type PHM 26). The pH constant apparatus is shown diagramatically in Fig. 1. As in the previous section, aliquots (15 ul) were removed at the onset and at intervals during the incubation, centrifuged and the supernatants analyzed for calcium and phosphate. At each time of sampling, the acid or base needed to keep the pH constant was recorded.

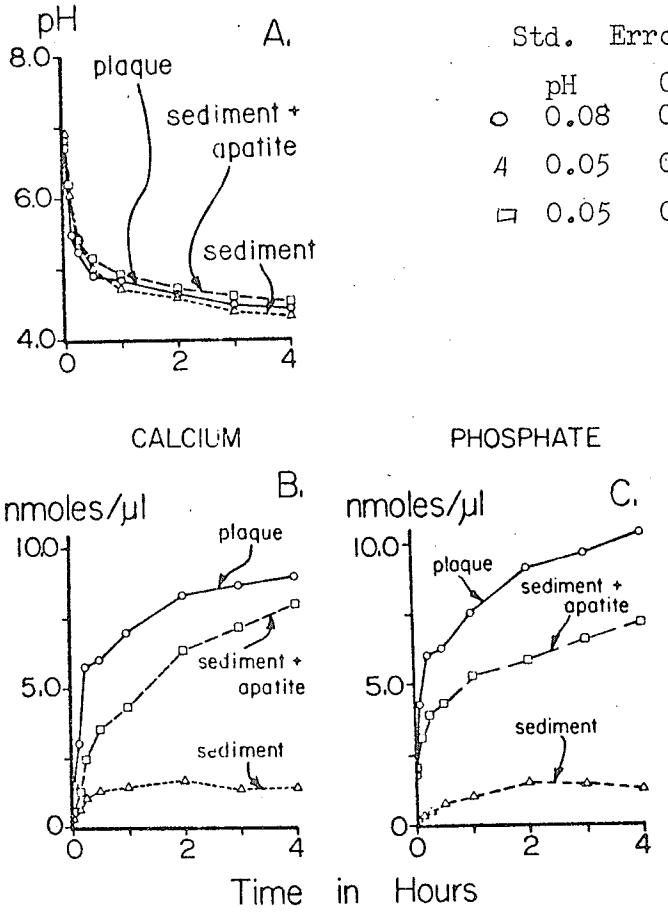
The same experiments were carried out with suspensions of hydroxyapatite or dicalciumphosphate (2.0 mg in 600 ul distilled water), i.e., without plaque or sediment present. The pH was also held constant at either of 4, 5, 6, 7, 8 or 9 and aliquots (15 ul) were removed at intervals for calcium and phosphate determinations. The amount of acid or base needed to maintain the pH constant during each incubation was also recorded.

(c) Effect of pH Progressively Lowered by the Addition of HCl and Lactic, Acetic or Propionic Acid

Four sediment mixtures, two with and two without 2.0 mg hydroxyapatite, were prepared containing 200 ul of 50 per cent sediment suspension and 400 ul deionized water. The pH of each was lowered by successive additions of either 0.1 N HCl or 0.1 N lactic, acetic or propionic acid during a 4 hour incubation. The amounts and times of the acid additions were so as to alter the pH in the same way as if the mixture was incubating with 0.5 per cent glucose. The experiments were carried out at 37°C. At intervals of 0, 7.5 and 15 minutes and 1, 2, 3 and 4 hours, aliquots (15 ul) were removed, and as in the previous sections, centrifuged and the supernatant analyzed for calcium and phosphate.

RESULTS

After preliminary experiments were completed, all plaque incubations were carried out twice; all others were carried out three times.



Std. Error of Means.

	pH	Ca	P.
o	0.08	0.16	0.19
Δ	0.05	0.06	0.06
□	0.05	0.18	0.23

Figure 2. The pH, calcium and phosphorus levels in plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM glucose. Calcium and phosphorus levels are expressed in nmoles/µl of supernatant.

Std Error of Means.

	pH	Ca	P
•	0.07	0.04	0.07
o	0.04	0.02	0.07
×	0.03	0.01	0.13

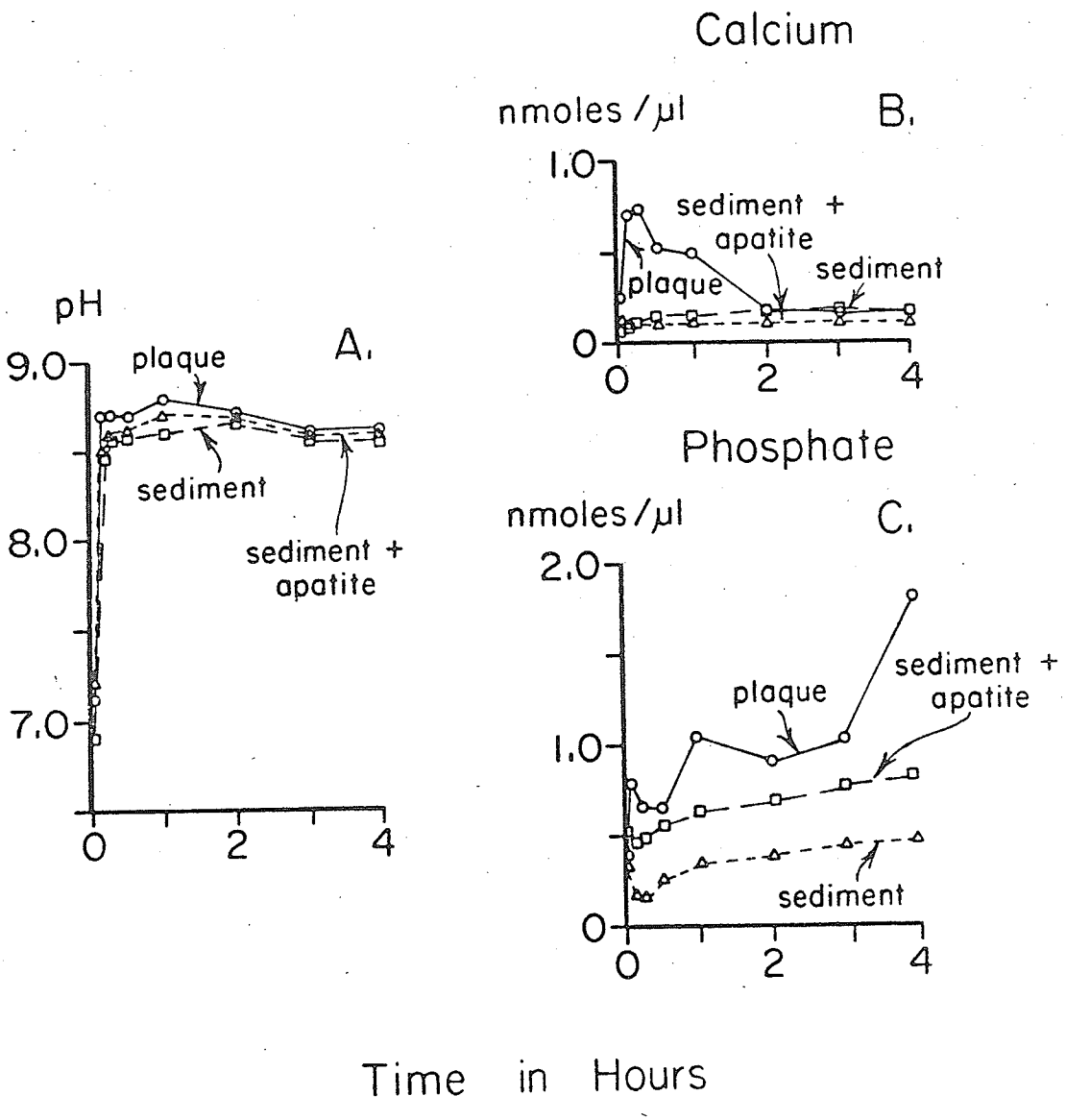


Figure 3. The pH, calcium and phosphate levels in plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM urea. Calcium and phosphate levels are expressed in nmoles of supernatant.

pH Changes and Calcium and Phosphate Released During Incubation of Plaque and Sediment Mixtures with (i) Glucose or (ii) Urea

With both glucose and urea, the pH of the plaque or sediment mixtures changed the most during the first half hour of incubation (Figs. 2 and 3). Both with sediment alone and with plaque, the differences in pH were small with glucose (Fig. 2) and undetectable with urea (Fig. 3). Analysis of variance showed statistically significant group differences (between plaque, sediment and sediment + hydroxyapatite) in the pH fall with glucose ($P < 0.01$) and pH rise with urea ($P < 0.05$). Addition of hydroxyapatite or dicalciumphosphate to the mixtures containing sediment reduced the pH decrease with glucose and the pH rise with urea (Figs. 2, 3 and 4). The effect was more with glucose than with urea.

The decrease in sediment pH was larger with 28 mM glucose than with 2.8 mM glucose but both hydroxyapatite and dicalciumphosphate when added inhibited the pH fall to a similar extent (approximately 0.2 pH units). The inhibition of pH fall by dicalciumphosphate was statistically significant ($P < 0.05$) for both 2.8 mM glucose and 28 mM glucose and by hydroxyapatite for 28 mM glucose. With 28 mM urea the pH rose rapidly and remained unchanged for most of the incubation. When 2.8 mM urea was utilized the pH rose less and fell to pre-incubation levels within the first hour. In both cases, either hydroxyapatite or

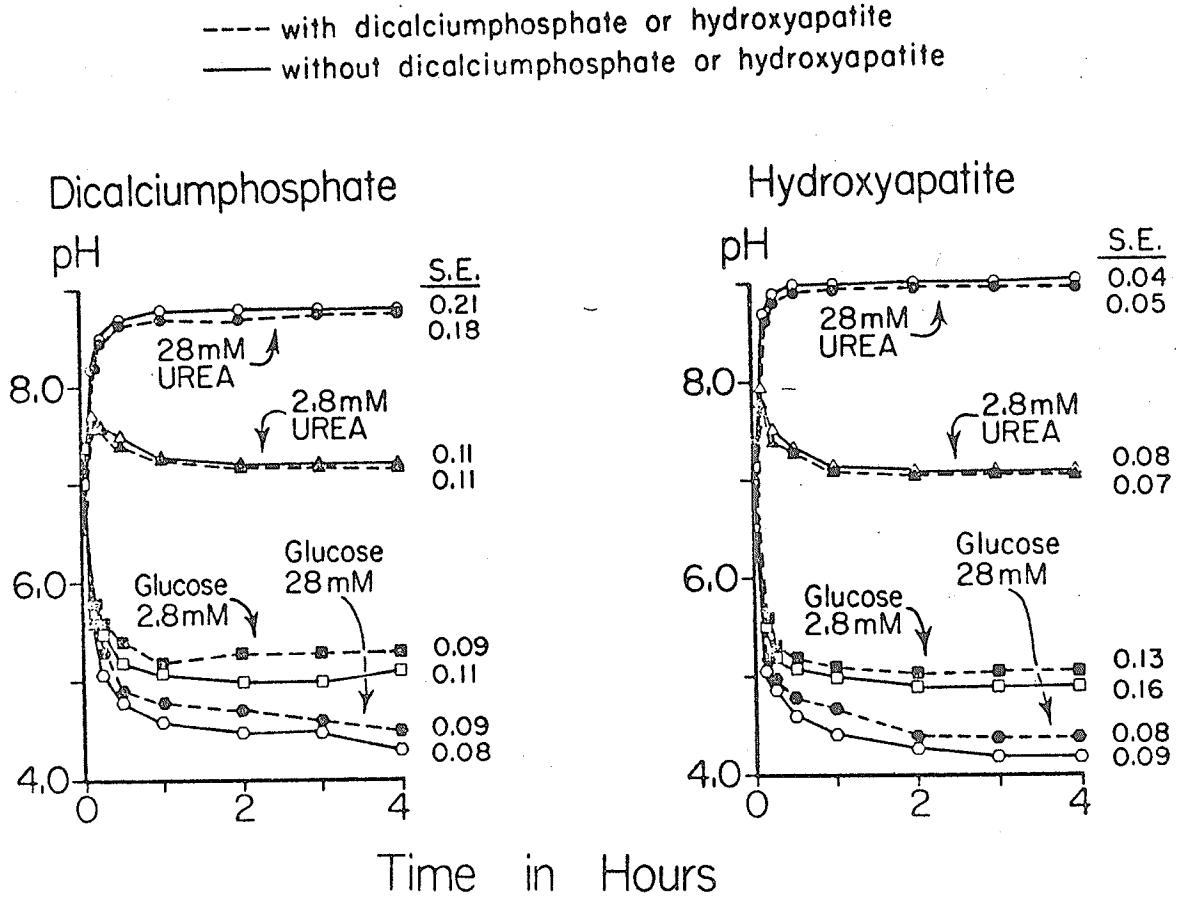


Figure 4. The pH of sediment, and sediment plus either hydroxyapatite or dicalciumphosphate incubated with 2.8 mM and 28 mM glucose and 2.8 mM and 28 mM urea. S.E. = Std Error of Means.

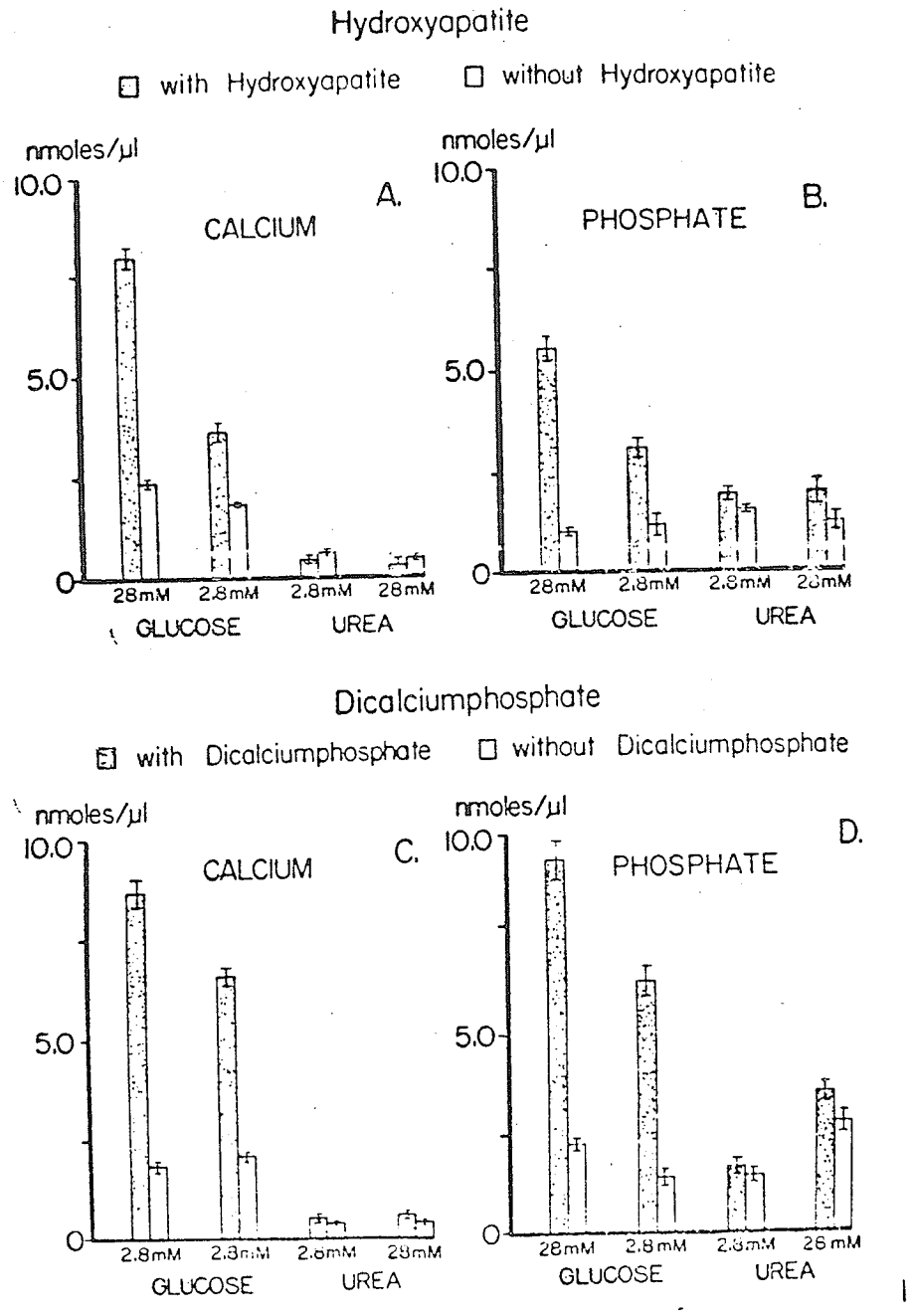


Figure. 5. Changes after 4 hours in the levels of calcium and phosphate released into the supernatant from sediment and sediment plus hydroxyapatite or dicalciumphosphate mixtures incubated with 2.8 mM and 28 mM glucose and 2.8 mM and 28 mM urea.
(I) = Std Error of Means.

dicalciumphosphate inhibited the extent of the pH rise slightly although this was not statistically significant.

When the substrate was glucose substantial amounts of calcium and phosphate were released both from plaque (Figs. 2b and 2c) and in the mixtures containing sediment plus hydroxyapatite or dicalciumphosphate; however, much smaller amounts of calcium and phosphate were released in the mixtures containing only sediment (Figs. 2, 5). Analysis of variance showed statistically significant group differences (between plaque, sediment and sediment + hydroxyapatite) in the calcium and phosphate release with 28 mM glucose and 28 mM urea ($P<0.01$).

More calcium than phosphate was solubilized with hydroxyapatite than with dicalciumphosphate (Fig. 5). Presence of phosphate but little or no calcium at the end of 4 hours was evident more with 28.0 mM than with 2.8 mM urea. With urea as substrate, the amounts of calcium and phosphate released either with hydroxyapatite or dicalciumphosphate present in the mixtures were much less than with glucose (Figs. 2b and 5).

Solubilization of Calcium and Phosphate from Hydroxyapatite and Dicalciumphosphate During Incubation at Constant pH

The release of calcium in mixtures of hydroxyapatite or dicalciumphosphate decreased exponentially as the pH increased (Fig. 6). Phosphate showed a similar decrease with dicalcium-

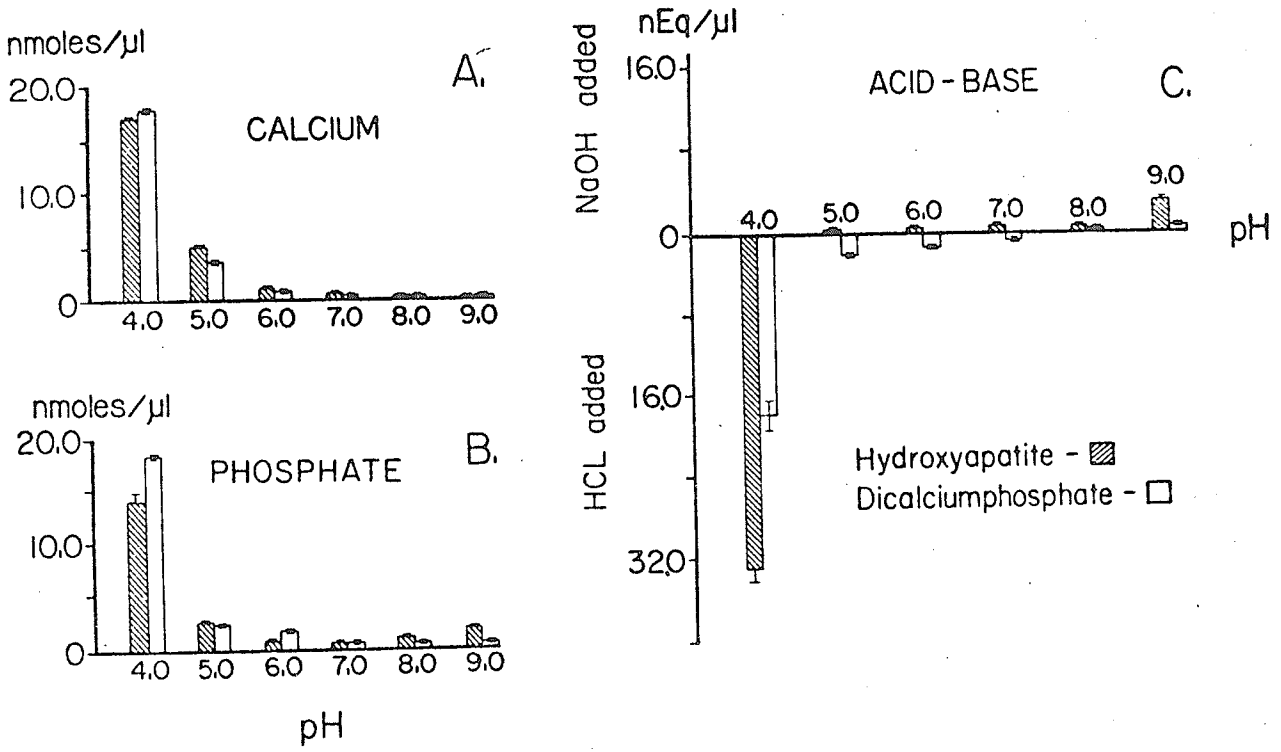


Figure 6. Changes after 4 hours in calcium, phosphorus and acid-base in suspensions of hydroxyapatite and dicalciumphosphate held constant at various pH levels. Calcium and phosphate levels are expressed in nmoles/µl of supernatant. (I) = Std Error of Means.

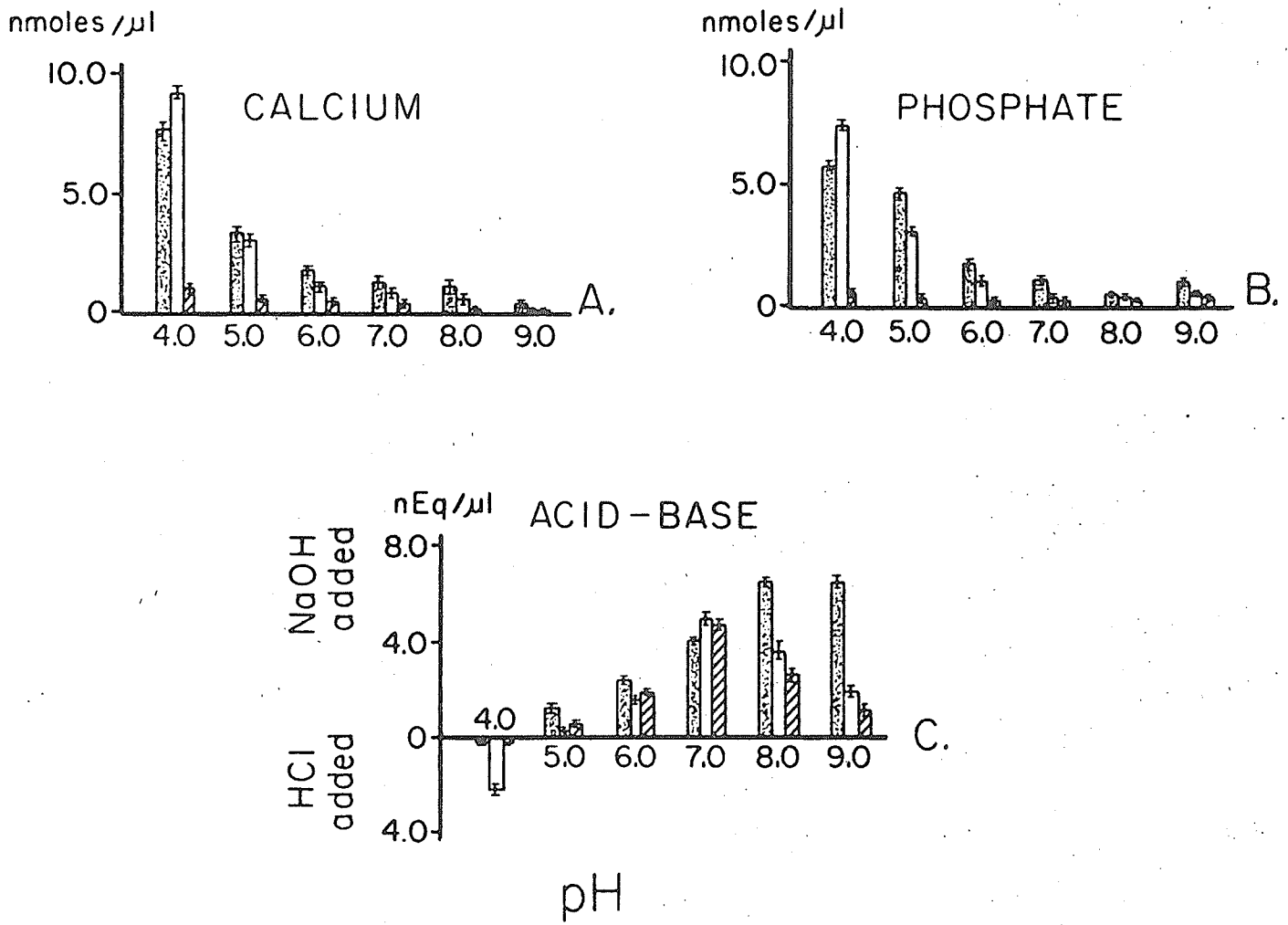


Figure 7. Changes after 4 hours in calcium, phosphorus and acid-base in plaque, sediment plus hydroxyapatite and sediment incubation mixtures held constant at various pH levels.

phosphate over the whole pH range but this was only true for hydroxyapatite below pH 7.0. Above this pH phosphate release then showed an increase. In both cases, acid was consumed at the acidic end of the pH range, (evident by the requirement to add HCl to keep the pH constant) whereas, base was consumed in the alkaline end of the pH range (evident by the need to add NaOH).

A plot of the logarithm of the calcium and phosphate values against pH for both salts is shown in Figs. 8a and 8b.

Release of Calcium and Phosphate During Incubation of Sediment and Plaque Mixtures at Constant pH

In mixtures containing either plaque alone or sediment plus hydroxyapatite the amount of calcium released decreased progressively with increase in the pH between 4 and 9 (Fig. 7). The changes with sediment were much less but showed a similar trend. Phosphate showed a smaller range of change. Less phosphate than calcium was solubilized at acidic but more was solubilized at alkaline pH. As in the previous section, acid was consumed in the acidic region of the pH range examined, whereas, base was consumed in the alkaline region.

A plot of the logarithm of the calcium values against pH is shown for plaque, sediment and sediment plus hydroxyapatite in Fig. 8c. A similar plot of the phosphate values is shown in Fig. 8d.

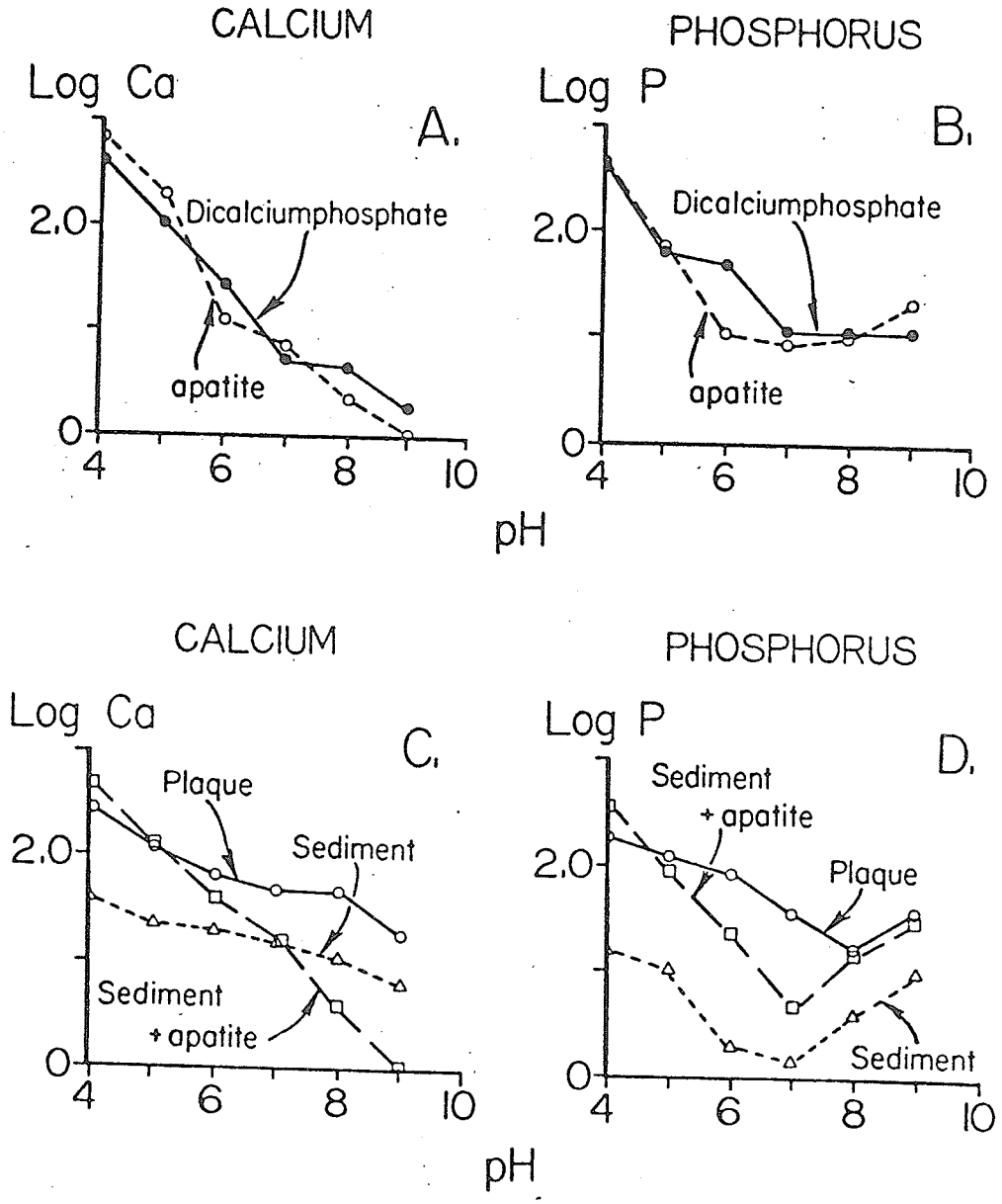


Figure 8. Release of calcium and phosphate into the supernatant from plaque, sediment plus hydroxyapatite, hydroxyapatite and dicalciumphosphate at constant pH. The logarithm of the calcium and phosphate concentration in ug/ml is plotted against pH.

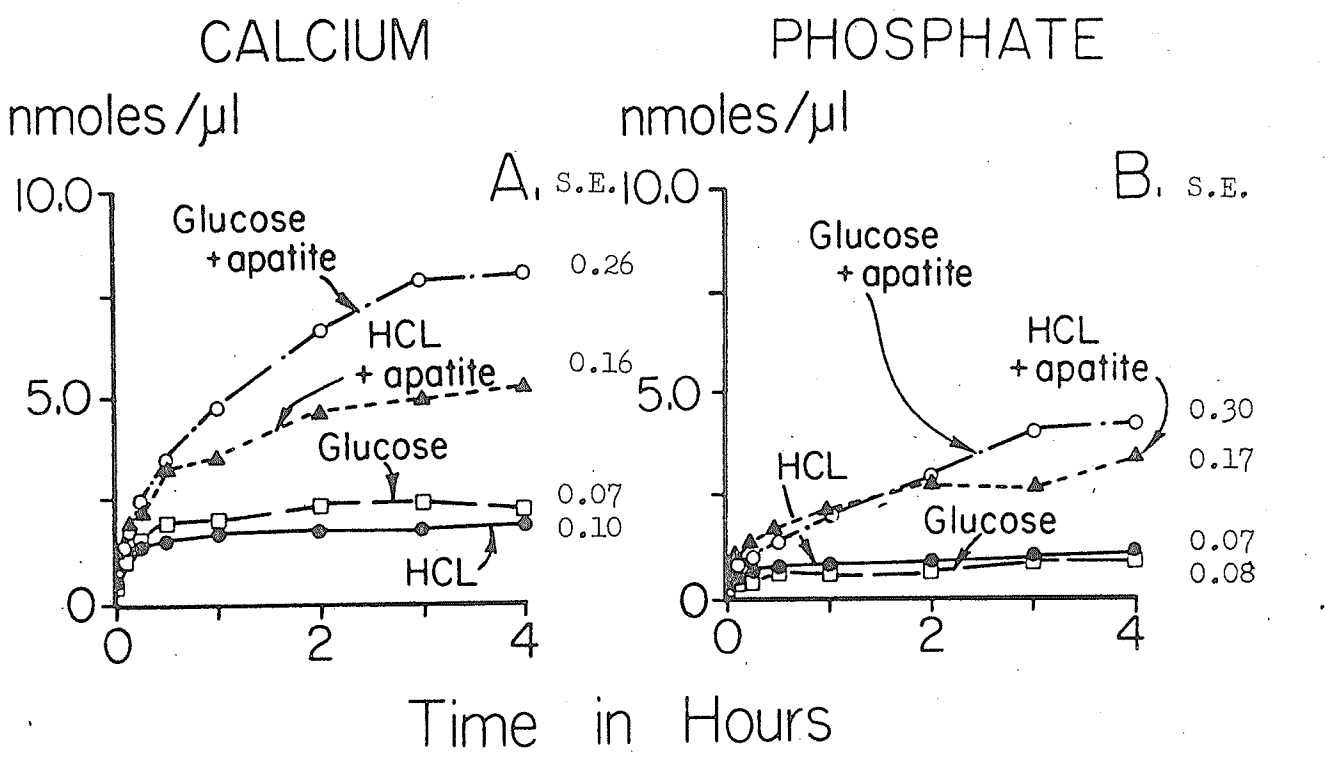


Figure 9. Changes during 4 hours in calcium and phosphate in sediment mixtures with and without hydroxyapatite resulting from lowering the pH by acid (1) formed during the incubation with 28 mM glucose and (2) by HCl added as though formed during the incubation with 28 mM glucose. Calcium and phosphate levels are expressed as nmoles/µl of supernatant.
S.E. = Std Error of Means.

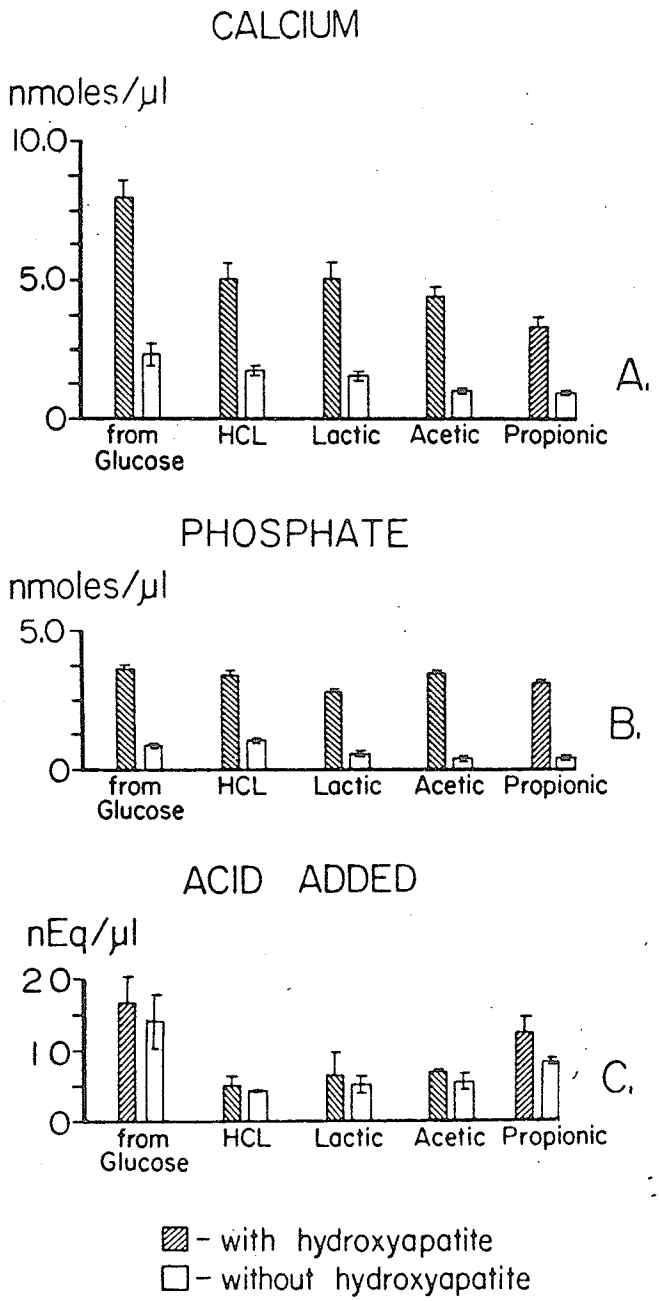


Figure 10. Changes after 4 hours in calcium, phosphate and acid-base levels in sediment mixtures with and without hydroxyapatite resulting from lowering the pH by acid (1) formed during the incubation of glucose and (2) added as though formed during such incubation. Calcium and phosphate levels are expressed in nmoles/µl of supernatant. (I) = Std. Error of Means.

Effect of Various Acids on Release of Calcium and Phosphate

Larger amounts of calcium were released upon incubation with glucose than in the incubations where the various acids were added. This held true whether or not hydroxyapatite was present (Fig. 9 and 10a).

The buffering capacity of the hydroxyapatite was evident in all cases since more acid was needed to obtain similar decreases in the pH when hydroxyapatite was present in the sediment mixtures.

More acid was present after incubation with glucose than was required to lower the pH and more calcium was solubilized with HCl and lactic acid than with the other acids (Fig. 10a). Very little difference was apparent in the phosphate solubilized during incubation with glucose or when acid was used to lower the pH (Fig. 10b).

DISCUSSION

As the pH decreased during the incubation of plaque with glucose, large amounts of plaque calcium and phosphate were solubilized. The amounts released were at least as much as that released in the sediment mixtures incubated with glucose and which contained either hydroxyapatite or dicalciumphosphate. On the other hand, the amounts released during the incubation of plaque or sediment with urea were considerably less than those with glucose and the pattern of release was quite different.

After an initial small increase, the concentration of calcium in solution slowly decreased. The phosphate concentration, on the

other hand, increased continuously throughout the incubation. The initial solubilization of calcium occurred as the pH was rising and the pH during this time was within a range where calcium phosphate is still soluble. Once the high pH was reached precipitation occurred. Interestingly, more solubilization occurred with plaque than with sediment plus hydroxyapatite suggesting that the calcium phosphate in plaque is more soluble than hydroxyapatite.

Although a previous study (Dawes and Jenkins, 1962) has indicated that washing plaque in water removes much of the inorganic phosphate, the results of this study have shown the release of substantial amounts of phosphate from plaque. Presumably much of it is bound but is released under acidic or basic conditions.

An amorphous calcium phosphate has been identified in plaque which may consist of poorly formed calcium phosphate and/or calcium and phosphate associated with carbohydrate-protein. It appears to be similar to that which precipitates from saliva along with carbohydrate-protein (Kleinberg et al., 1971) and which *in vitro* experiments indicate is easily solubilized.

In the constant pH experiments, the relation between the logarithm of the calcium released and the pH was linear. This held true for hydroxyapatite and dicalciumphosphate alone, for sediment and plaque, and for sediment plus added hydroxyapatite (Fig. 8). The slopes for sediment and plaque were similar (about 0.2), but each was steeper than the slopes for hydroxyapatite, dicalciumphosphate or sediment plus hydroxyapatite. The latter

group were about the same and equal to about 0.5. A similar interrelationship between the logarithm of the calcium concentration and pH has been observed in previous studies (Hodge 1951, Levinskas and Neuman, 1955).

Sediment without added calcium phosphate showed the same effects of pH and patterns of release of calcium and phosphate that were seen with plaque but the magnitudes of the effects were much less. This can be explained if the sediment, which arises from centrifuged saliva, contains some of the amorphous calcium phosphate believed to be deposited during plaque formation from saliva (Kleinberg et al., 1971).

In those experiments where the pH was lowered by the addition of either HCl or the major acids formed when glucose is catabolized by either plaque or sediment, calcium appeared to be affected more than phosphate by the type of acid. Release of phosphate showed little difference. HCl was needed in least amount to lower the pH, yet it dissolved the greatest amount of calcium. This can be attributed to HCl being highly dissociated. As a result, less acid would be required to lower the pH than a less dissociated acid; furthermore, more anion would be available for association with calcium. Thus, calcium solubilization by acid, as pointed out by Buonocore (1963), would depend upon the dissociation constant of the acid and the formation constant of its anion with calcium.

More acid formation and more calcium solubilization occurred

when sediment was incubated with glucose. One explanation for a larger quantity of acid being required to attain the same pH as is obtained by simply adding acid, could be that during the incubation, some base is formed along with the acid (Korayem and Kleinberg, 1970; Kleinberg, Craw and Komiyama, 1973).

It is interesting to note that these results are in general agreement with those of Geddes (1972) for plaque, which showed that the addition of equivalent amounts of individual organic acids to plaque samples produced different pH decreases with lactate producing a lower pH than acetic or propionic acids.

Interaction Between Changes in Acid-Base and Calcium Phosphate Solubility

That calcium phosphate is more soluble at acidic than at neutral or alkaline pH was evident in all the experiments in this study whether or not changes in pH were brought about as a result of cellular metabolism or simply by the addition of acid or base. When calcium and phosphate were solublilzed at acidic pH, acid was consumed, whereas, at alkaline pH, as phosphate (and little or no calcium) was solubilized, base was consumed. These changes were particularly evident in the constant pH experiments, and may be explained as follows.

When hydroxyapatite dissolves at slightly acidic pH and dissolution is not complete, a salt of lower calcium to phosphate

ratio than hydroxyapatite and one of greater solubility is formed (Gray, Francis and Griebstein, 1962).

$$Ca_{10}(PO_4)_6(OH)_2 + 8\ H^+ \rightleftharpoons 6\ CaHPO_4 + 4\ Ca^{++} + 2HOH \quad (1)$$

$$CaHPO_4 \rightleftharpoons Ca^{++} + HPO_4^{=} \quad (2)$$

The H^+ in solution essentially exchanges for the Ca^+ in the hydroxyapatite and is taken up by $PO_4^{\equiv}$ which becomes $HPO_4^{=}$ either as part of solid $CaHPO_4$ or as ions in solution (equations (1) and (2) respectively and see Gray et al., 1962). At a pH of 4.0, dissolution would be extensive and the H^+ would be consumed as $PO_4^{\equiv}$ is converted not only to $HPO_4^{=}$ but also to $H_2PO_4^{-}$. If dicalciumphosphate replaced the hydroxyapatite, H^+ would be utilized in converting $HPO_4^{=}$ to $H_2PO_4^{-}$.

At alkaline pH, hydroxyapatite is converted to a less soluble salt or forms a surface phase, either one being of higher Ca/P ratio (Rootare, Deitz and Carpenter, 1962).

$$Ca_{10}(PO_4)_6(OH)_2 + 8HOH + 2OH^- \rightleftharpoons 5\ Ca_2HPO_4(OH)_2 + HPO_4^{=} + 2HOH \quad (3)$$

$$Ca_2HPO_4(OH)_2 \rightleftharpoons 2\ Ca^{++} + HPO_4^{=} + 2OH^- \quad (4)$$

Under these conditions OH^- in solution exchanges for phosphate and since $Ca_2HPO_4(OH)_2$ has poor solubility, equation (4) is not likely to be extensive. Thus, phosphate (equation 3) but little calcium would be released as was observed in the present study (Fig. 3b, 5a and 5c).

Equations (1) and (3) are essentially transformation reactions and show how hydroxyapatite can act both as an acid-base buffer and as a regulator of the Ca/P ratio of the medium. At acidic pH more solubilization occurs and leads to more buffering than at alkaline pH (Fig. 4). Further, preferential release of calcium at acidic pH favours an increase in the Ca/P ratio; whereas, the preferential release of phosphate at alkaline pH favours a decrease in the ratio.

CHAPTER IV

EFFECT OF FLUORIDE ON THE pH AND RELEASE OF CALCIUM AND PHOSPHATE FROM DENTAL PLAQUE IN VITRO

INTRODUCTION

Dental caries reduction by fluoride is well established; however, the mechanisms involved are still not fully understood. Fluoride may reduce dental caries by inhibiting the formation of acid along with several other parameters of the carbohydrate metabolism of the oral microorganisms (glucose uptake, Sandham and Kleinberg, 1969; Weiss et al., 1965; Hamilton, 1969b; fructose and sucrose uptake, Halhoul, 1972; formation of storage polysaccharide, Sandham and Kleinberg, 1969; Hamilton, 1969a). This inhibition is most effective when the pH falls below approximately 5.0 (Jenkins, 1959).

Fluoride may also reduce dental caries by decreasing the solubility of tooth mineral. This may occur in several ways. Firstly, fluoride favours the more crystalline, less soluble forms of calcium phosphate (Frazier and Engen, 1966). Secondly, fluoride favours the formation of larger crystals and therefore a smaller available surface area for solubilization (Posner et al., 1963). Finally, the rate of calcium phosphate dissolution may be reduced either directly or indirectly by fluoride (Rathje, 1952; McCann and Bullock, 1957).

Plaques from individuals residing in low and high fluoride areas and showing large differences in plaque fluoride levels show only minute differences in the minimum pH upon incubation with sucrose (Edgar et al., 1970). One explanation may be that the plaque fluoride although at high levels in plaque is present mostly in bound form (Jenkins and Edgar, 1969).

Plaque also contains high levels of calcium and phosphate, both of which are solubilized when acid is formed during the bacterial breakdown of glucose (see previous chapter). Since their dissolution affects the pH and fluoride may affect their solubilization, experiments were carried out to determine the effect of fluoride on calcium and phosphate release from or retention by plaque.

Therefore, experiments were carried out to examine the effect of fluoride on the release of calcium and phosphate in plaque mixtures in which the pH was varied in a number of ways within the pH range of 4.0 to 9.0. As previously (Chapter III) and for the same reasons, similar experiments were carried out with mixtures containing salivary sediment, sediment plus added hydroxyapatite and suspension of hydroxyapatite. In one series of experiments, the pH of sediment and plaque mixtures were altered metabolically by incubation with either glucose or urea; the former produces a decrease in pH from neutrality to about

4.0 and the latter causes the pH to rise to about 9.0. In another series, the effect of fluoride on calcium and phosphate release during the metabolism of glucose in plaque and salivary sediment mixtures in which the pH was held constant at each of several pH levels between 4.0 and 9.0 was also examined. Similar constant pH experiments were carried out to determine the effect of fluoride on the solubilization of calcium and phosphate from hydroxyapatite alone.

METHODS

Preparation of Plaque and Salivary Sediment Incubation Mixtures

Plaque and salivary sediment were collected and prepared as in the previous chapter. Mixtures (600 ul) of either were prepared with the following components and final concentrations (i) plaque at 8.3 per cent (V/V) or sediment at 16.7 per cent (V/V) (ii) glucose or urea at 28 mM (iii) fluoride at 0 or 5 ppm and (iv) either no hydroxyapatite or 2.0 mg of this salt. The hydroxyapatite used in this study was prepared as described in the previous chapter.

An earlier study had shown that plaque and sediment suspensions at 8.3 per cent and 16.7 per cent respectively produce similar pH changes with glucose and urea (Singer and Kleinberg, 1970); therefore, comparisons were made at these concentrations.

Incubation Procedures

In the first series of experiments, the effects of fluoride on calcium and phosphate release from mixtures containing plaque (2), sediment (2) or sediment plus hydroxyapatite (2) were examined during their incubation at 37°C for 4 hours using either glucose or urea as metabolites. Plaque was examined by itself and this was done twice. The sediment and sediment plus hydroxyapatite mixtures were studied together and done 3 times. At intervals during each incubation, the pH was measured and aliquots (15 ul) were removed and prepared for calcium and phosphate analyses as described in Chapter III (page 28 of this thesis).

In a second series of experiments plaque mixtures (2) or salivary sediment mixtures (4), 2 of the latter with and 2 without hydroxyapatite, were incubated with 0.5 per cent glucose. The mixtures were then incubated for 4 hours at a constant pH of either 4, 5, 6, 7, 8 or 9. The pH, calcium and phosphate solubilized and the acid or base needed to maintain the pH constant during the incubation were determined. Each experiment was carried out twice.

A third series of experiments examined the effect of 5 ppm fluoride upon the release of calcium and phosphate from hydroxyapatite (2.0 mg) suspended in distilled (600 ul) water and incubated at constant pH as in the experiments in the previous

paragraph. Saliva contains very low levels of fluoride (0.1 - 0.2 ppm, Martin and Hill, 1950) while plaque contains high levels (6 - 180 ppm, Hardwick and Leach, 1962). An intermediate level of 5 ppm fluoride was chosen for this study because it gave a detectable level of pH inhibition as well as a distinct suppression of calcium and phosphate release from plaque and sediment. These experiments were each carried out twice.

RESULTS

Effect of Fluoride on the pH Fall and Rise During Incubation with Glucose or Urea

The mixtures containing either plaque, sediment or sediment plus hydroxyapatite incubated with glucose showed a rapid fall in the pH during the first 30 minutes and only a small additional decrease thereafter (Fig. 11). Fluoride inhibited this pH fall, more in sediment than in plaque; however, in the sediment mixtures containing added hydroxyapatite it stimulated the pH fall slightly; analysis of variance showed statistical significance of this inhibition for sediment ($P < 0.01$) but not for plaque; stimulation of pH fall by fluoride for sediment plus hydroxyapatite was statistically significant ($P < 0.01$).

With urea, the pH of the same mixtures rose rapidly to a maximum within the first 15 to 30 minutes and then remained unchanged for the remainder of the 4 hour incubation (Fig. 12).

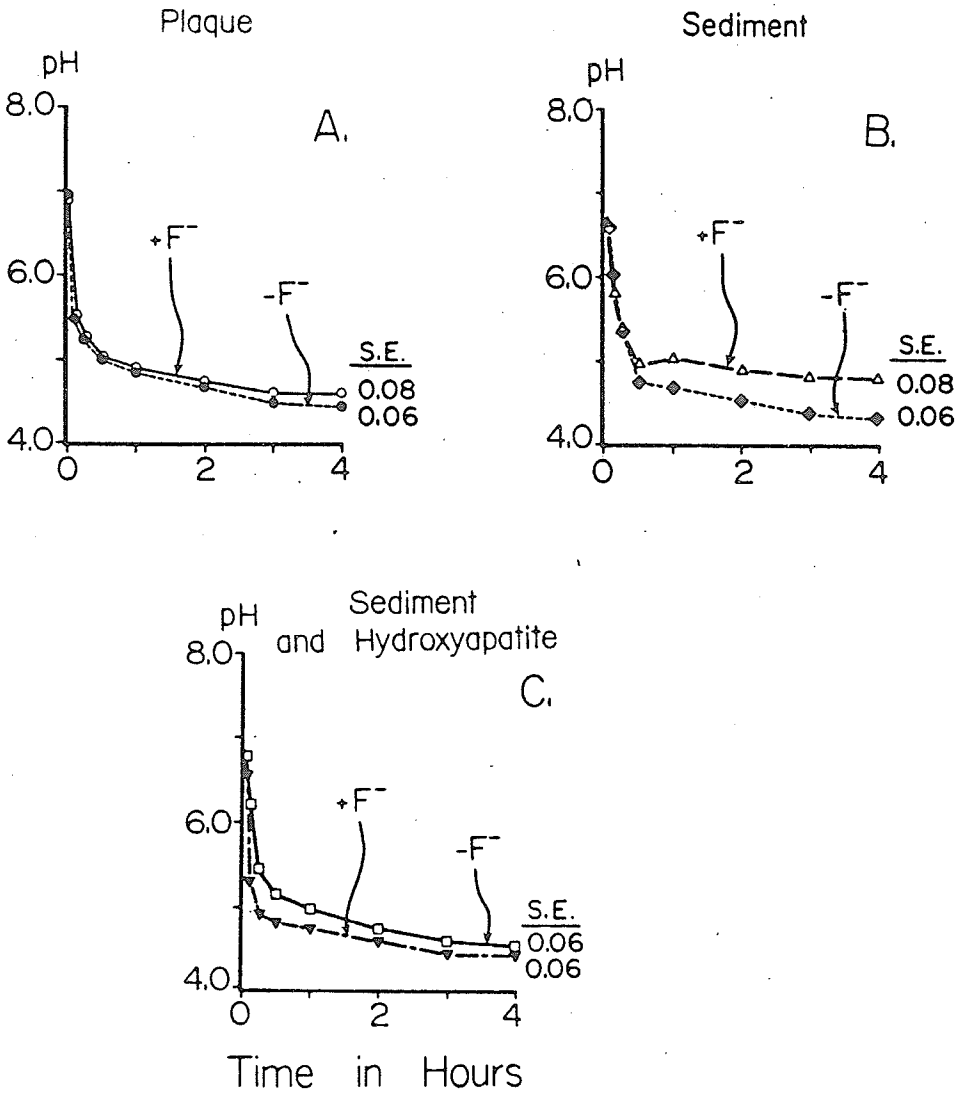


Figure 11. Effect of 5 ppm fluoride on the pH of plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM glucose.

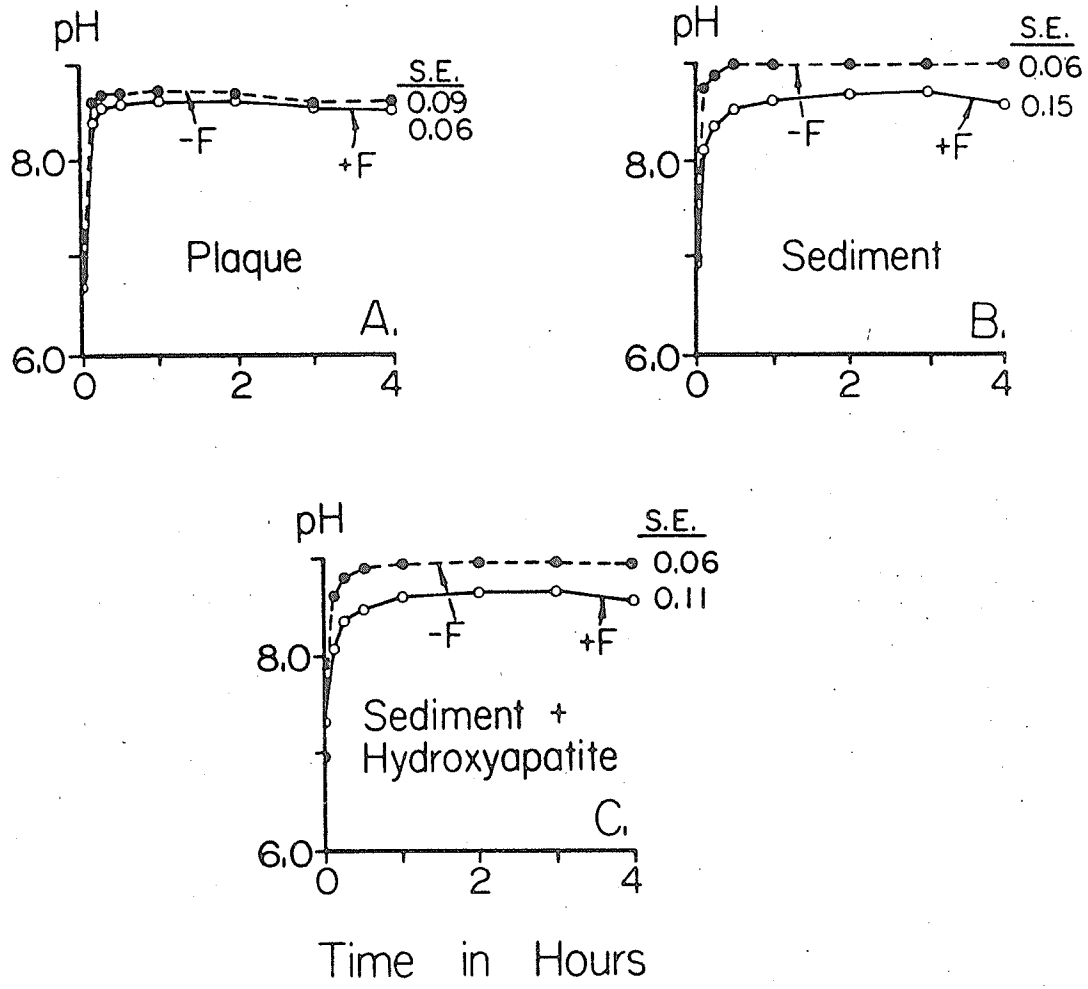


Figure 12. Effect of 5 ppm fluoride on the pH of plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM urea.

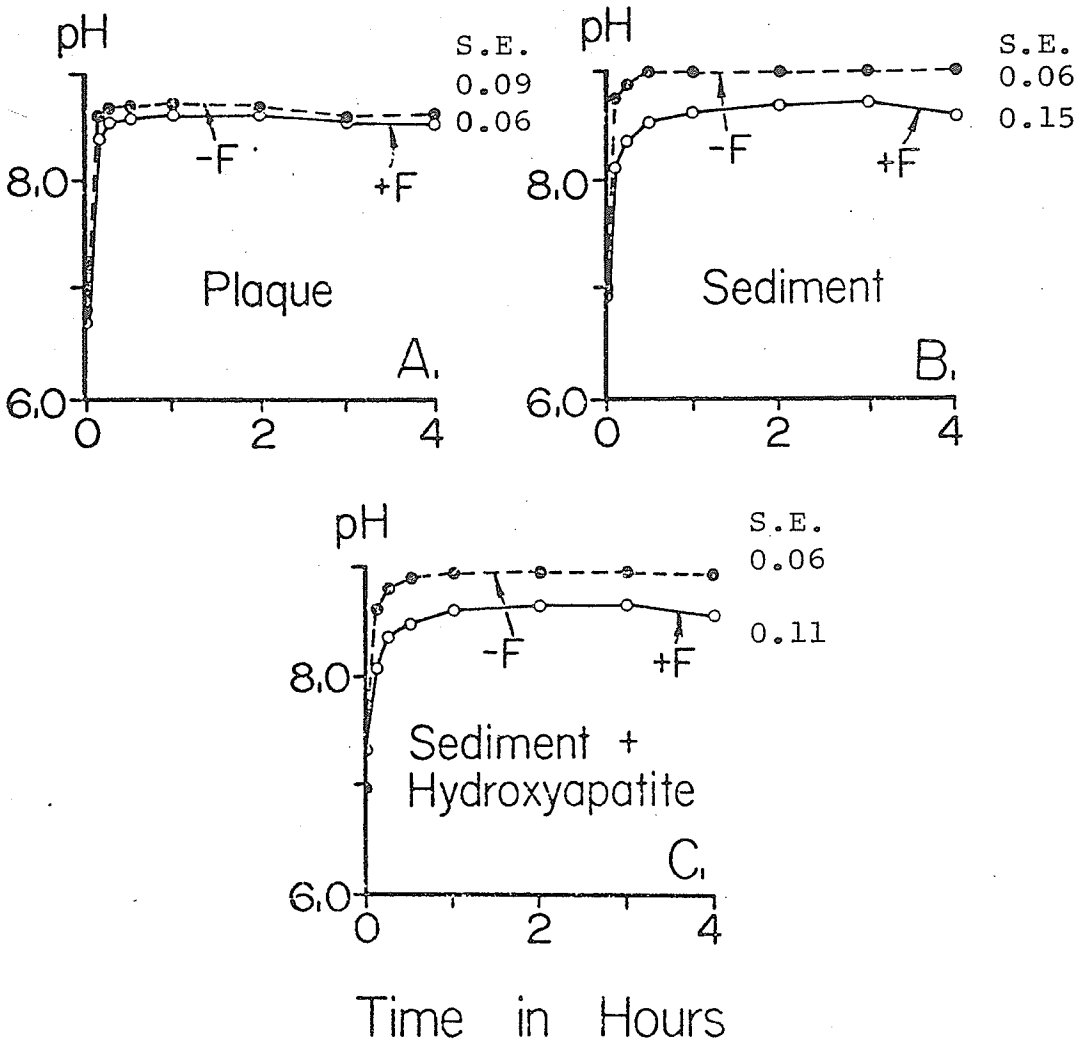


Figure 12. Effect of 5 ppm fluoride on the pH of plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM urea. S.E. = Std. Error of Means.

Fluoride inhibited the pH rise markedly in the mixtures containing sediment and sediment plus hydroxyapatite but had only a slight effect on the pH rise with plaque. Analysis of variance showed statistical significant of this inhibition of pH rise for plaque ($P<0.05$) and for sediment and sediment plus hydroxyapatite ($P<0.01$).

Effect of Fluoride on Calcium and Phosphate Release during Incubation with Glucose or Urea

In each of the three types of mixtures; plaque, sediment and sediment plus hydroxyapatite, fluoride suppressed the release of both calcium and phosphate by approximately 50 per cent when glucose was the substrate (Fig. 13); analysis of variance showed this inhibition to be statistically significant for plaque, sediment and sediment plus hydroxyapatite ($P<0.01$). When the substrate was urea, fluoride was without effect on calcium and phosphate release in the mixtures containing sediment, both in those with and in those without added hydroxyapatite. However, fluoride inhibited the initial release of calcium and the release of phosphate from plaque (Fig. 14), but these results were not statistically significant.

Effect of pH on Fluoride Inhibition of Calcium and Phosphate Release when the pH was Held Constant

Fluoride suppressed the release of calcium throughout the

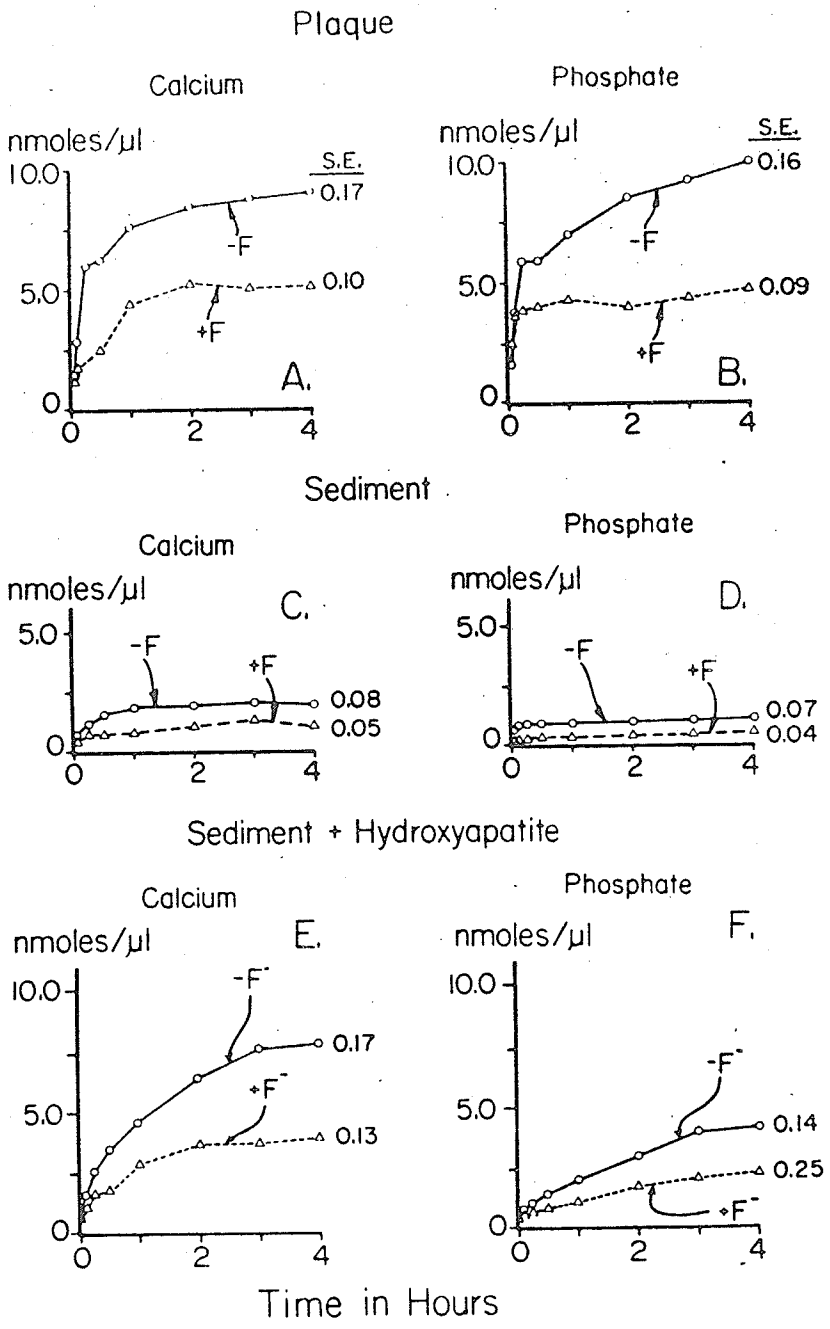


Figure 13. Effect of 5 ppm fluoride on calcium and phosphorus levels in plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM glucose.

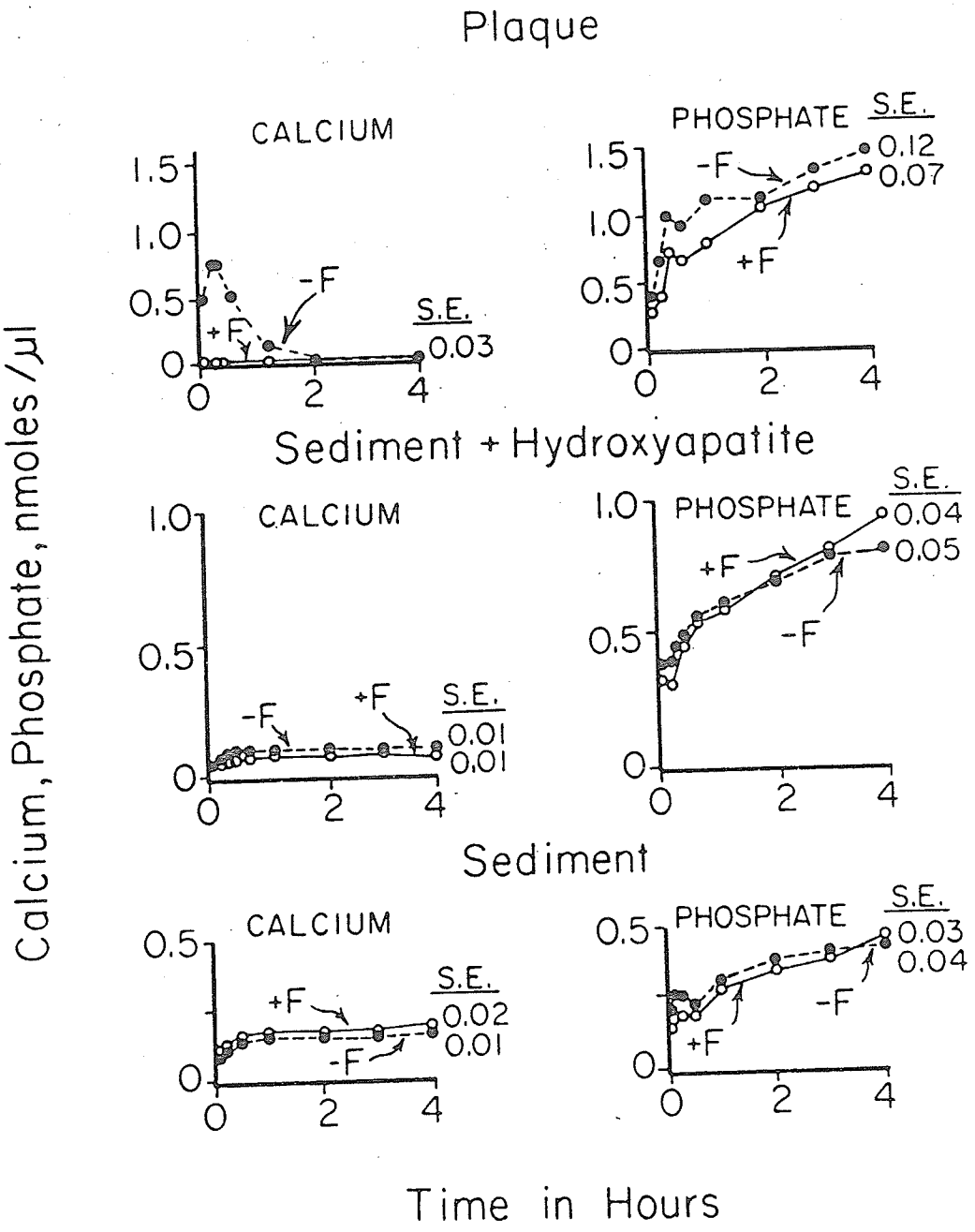


Figure 14. Effect of 5 ppm fluoride on calcium and phosphorus levels in plaque, sediment and sediment plus hydroxyapatite mixtures incubated with 28 mM urea.

entire pH range in each of the three different systems (Figs. 15a, 16a and 17a) and in hydroxyapatite mixtures without cells (Fig. 18a). Except around pH 7.0 and 8.0, fluoride also suppressed the release of phosphate (Figs. 15b, 16b and 17b). The results confirmed the relationships between pH and calcium and phosphate solubility in that (i) calcium and pH were inversely and exponentially related over the whole pH range (ii) phosphate was similarly related below pH 7.0 but at alkaline pH, the solubility of phosphate increased.

The acid or base required to maintain the pH constant in each of these experiments is shown in Figs. 15c, 16c, 17c and 18c. The results for plaque and sediment were similar; those for hydroxyapatite and sediment plus hydroxyapatite were similar. Both sets, in general, showed stimulation of acid formation, evident by the need to add base, at pH 5.0, 6.0, 7.0 and 8.0. The two sets, however, differed at pH 4.0 and 9.0. Sediment and plaque showed the need for less acid and base in the presence of fluoride. Hydroxyapatite and sediment plus hydroxyapatite needed more or both, respectively.

DISCUSSION

Fluoride Effects on pH

The results of the present study indicate that in addition to inhibiting the pH fall in both plaque and salivary sediment mixtures incubated with glucose, fluoride also inhibits the pH rise when the same mixtures are incubated with urea. However,

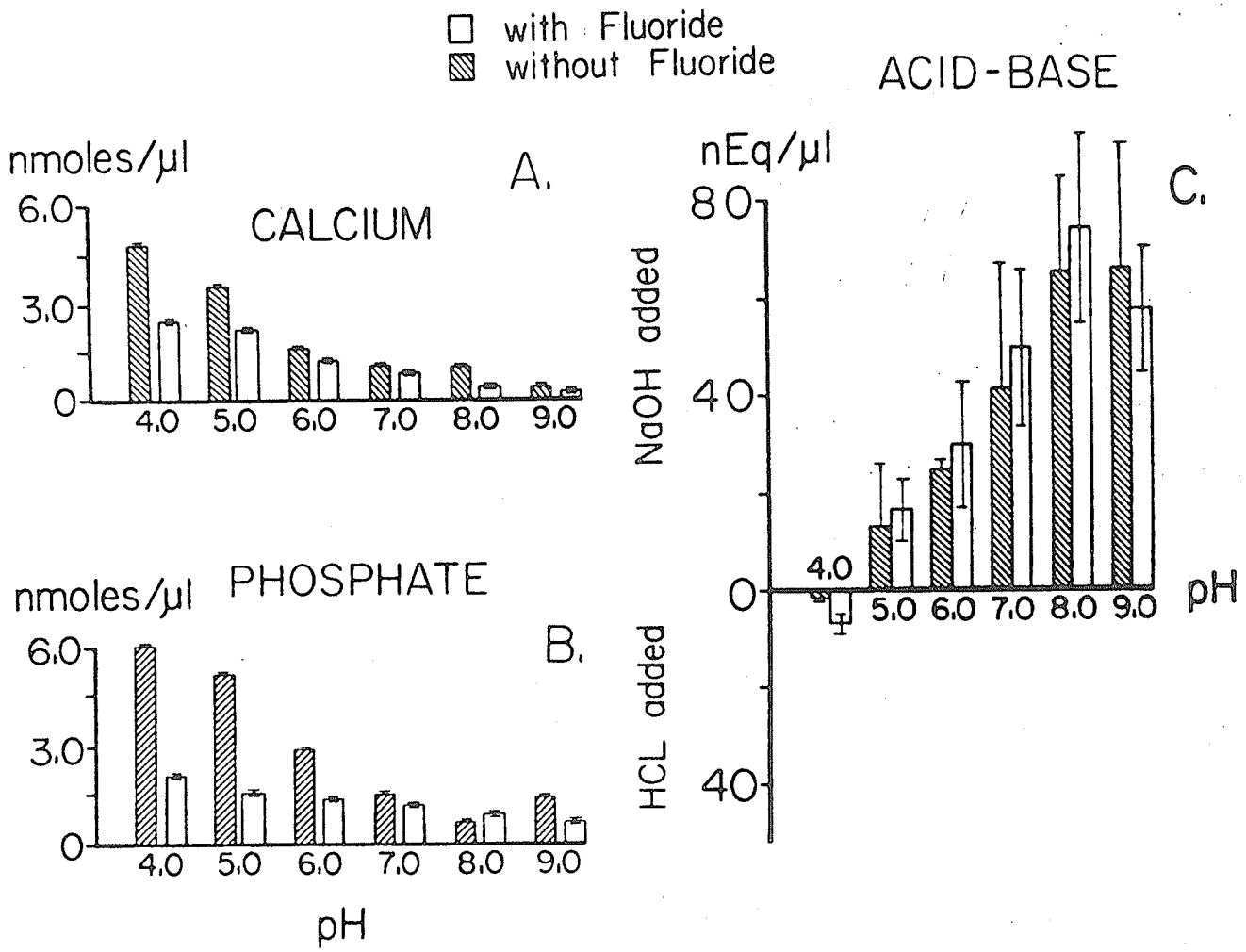


Figure 15. Effect of 5 ppm fluoride on changes after 4 hours in calcium, phosphorus and acid-base in plaque mixtures incubated with 28 mM glucose and the pH held constant at various levels.

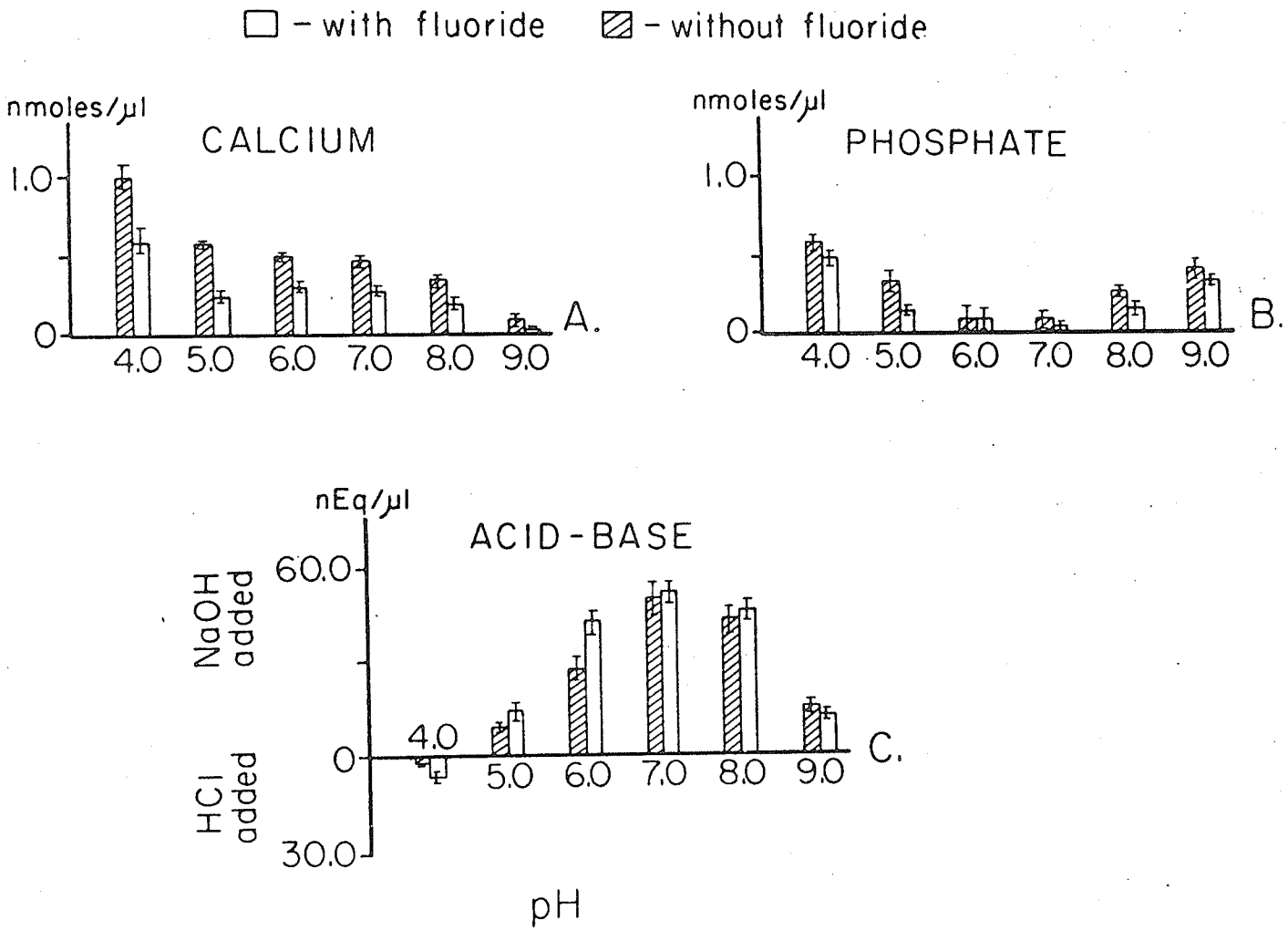


Figure 16. Effect of 5 ppm fluoride on changes after 4 hours in calcium, phosphorus and acid-base in sediment mixtures incubated with 28 mM glucose and the pH held constant at various levels.

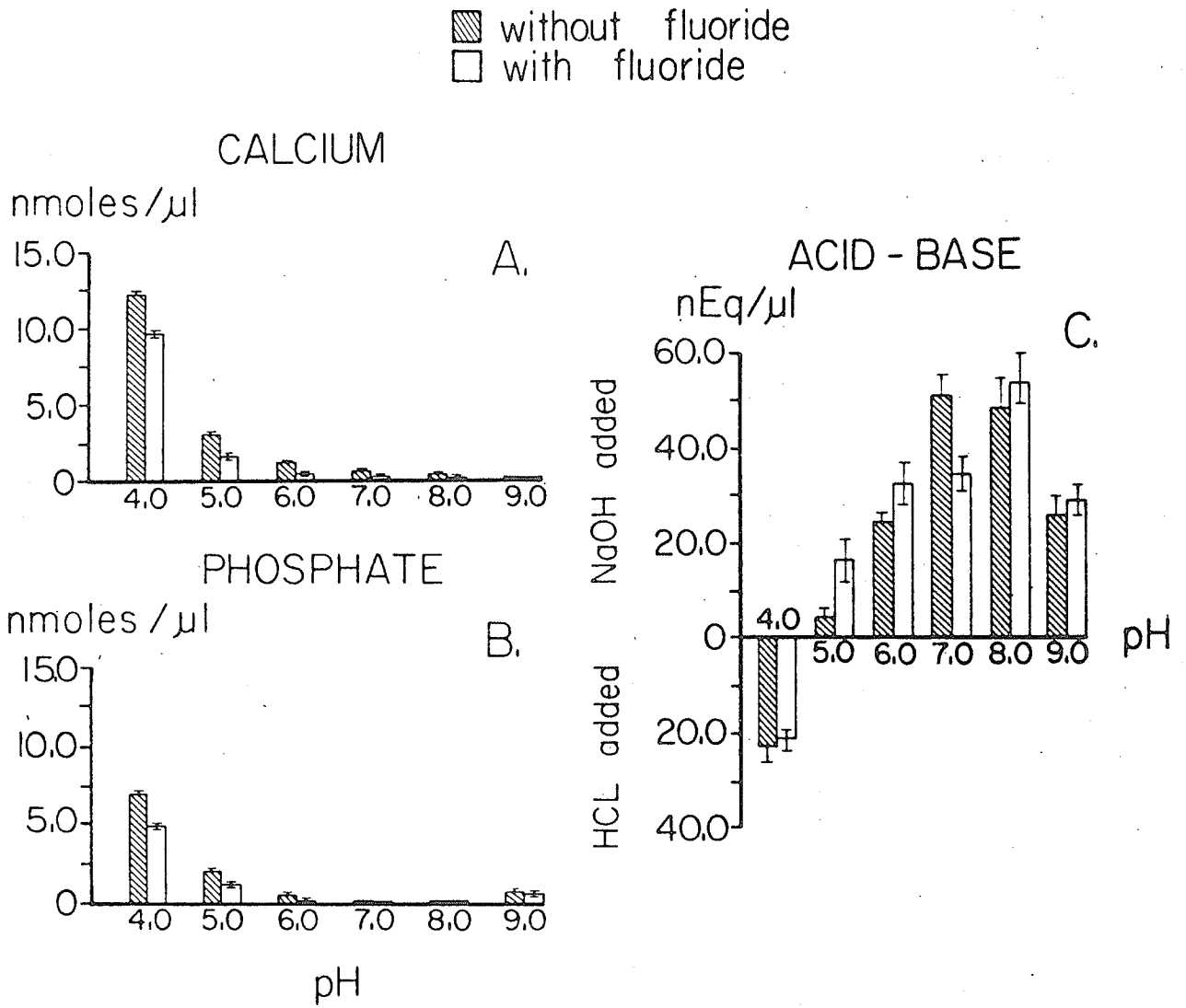


Figure 17. Effect of 5 ppm fluoride on changes after 4 hours in calcium, phosphorus and acid-base in sediment plus hydroxyapatite mixtures incubated with 28 mM glucose and the pH held constant at various levels.

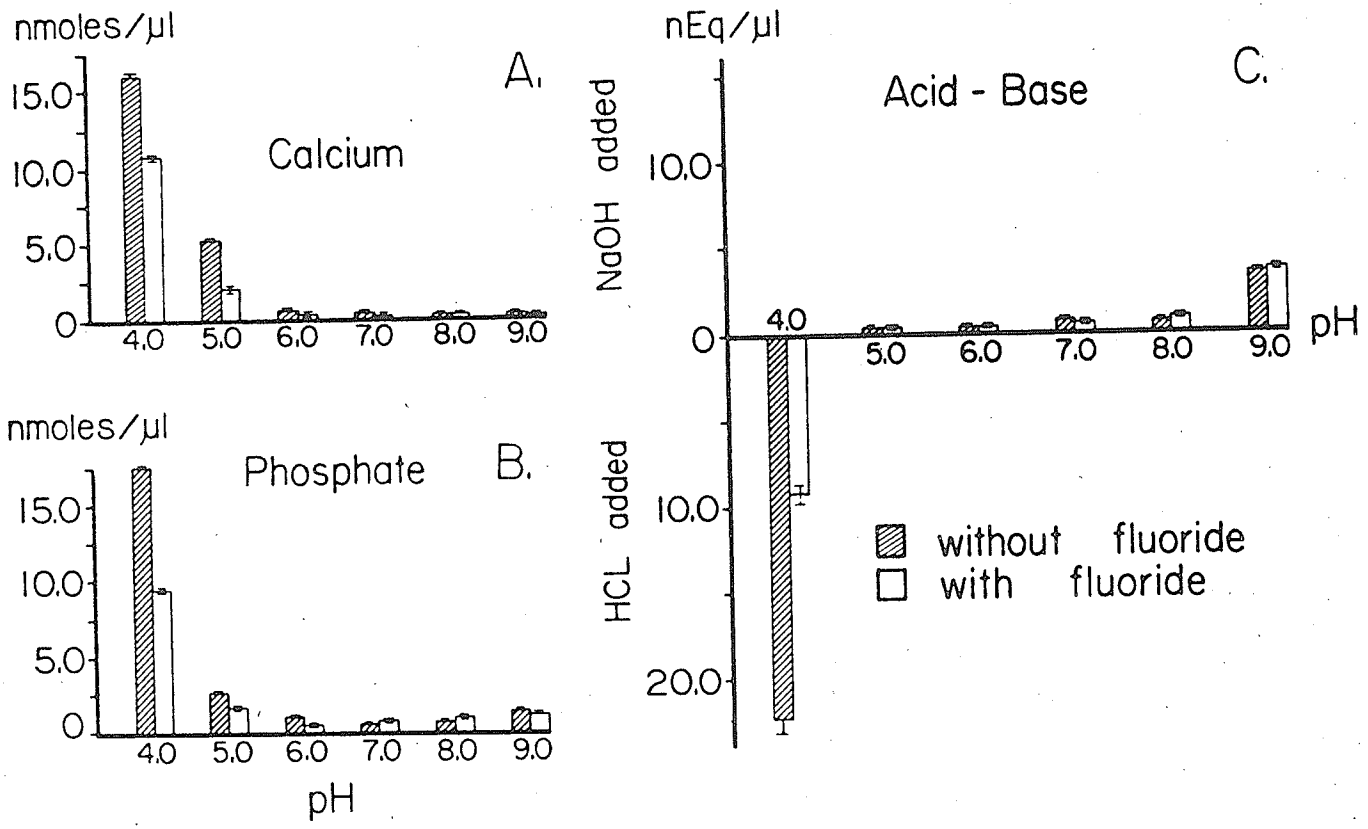


Figure 18. Effect of fluoride on calcium, phosphorus and acid-base changes in a suspension of hydroxyapatite held constant for 4 hours at various pH levels.

smaller effect for both substrates was observed with plaque. Addition of hydroxyapatite to sediment mixtures had no effect on fluoride inhibition when urea was the metabolite; on the other hand, it had a marked effect when the substrate was glucose. Here hydroxyapatite resulted in a greater fall in the pH than in those mixtures containing fluoride without added hydroxyapatite.

These complicated effects on the pH can be explained as follows. The pH falls to below 5.0 in both plaque and sediment mixtures incubated with glucose. This fall is the result of (i) the accumulation of acid during glycolysis and (ii) the neutralization of acid when hydrogen ions are used to dissolve the calcium phosphate present (Chapter III, page 29 of this thesis). Since fluoride inhibits glycolysis under these conditions, it would reduce the extent of the pH fall by reducing the amount of acid accumulating. On the other hand, by inhibiting solubilization of calcium phosphate, fluoride could increase the pH fall by reducing acid neutralization by the calcium phosphate. Thus, fluoride could affect the pH differently depending upon the magnitude of its effects on each or both of these processes.

In sediment, where calcium and phosphate levels are low, inhibition of the pH fall by fluoride was large and therefore for the most part would be by the inhibition of glycolysis. In plaque, however, where calcium and phosphate levels are high, inhibition of the pH fall was slight; this would occur if fluoride

inhibition of both acid formation and neutralization largely counteracted one another. In mixtures of sediment plus added hydroxyapatite the pH fall was stimulated by fluoride. When added under these conditions, fluoride would have access to the hydroxyapatite and if incorporated would result in no fluoride being available to inhibit glycolysis as in the mixtures containing sediment alone.

With urea (28 mM) the large fluoride effect on the pH rise in sediment with or without hydroxyapatite can be attributed to little calcium phosphate dissolution at alkaline pH. Thus, fluoride is available to inhibit urea catabolism. However, the absence of a fluoride effect on the pH with plaque may have occurred because some calcium phosphate solubilization occurred and the fluoride may have been incorporated into the salt as it was subsequently precipitated; therefore, the fluoride would not have been free to inhibit the urea metabolism (Fig. 14).

Effect of Fluoride on Calcium and Phosphate Release

Fluoride suppressed the release of both calcium and phosphate by about 50 per cent in plaque, sediment and sediment containing hydroxyapatite when each was incubated with 0.5 per cent glucose. Since little calcium is released when urea is metabolized, the fluoride is without obvious effect except with plaque, where it initially shows brief inhibition (Fig. 14), although this is

not of statistical significance. Fluoride does suppress the release of phosphate from plaque to a small extent but not from sediment or sediment with added hydroxyapatite. These results also occurred with hydroxyapatite alone indicating that the fluoride effects in plaque and sediment are primarily due to its action on the calcium and phosphate in these cellular systems.

The calcium and phosphate released from plaque and sediment probably originated from both the cells and the intercellular components of these systems.

Hydroxyapatite crystals have been observed within bacteria comprising the oral flora (Ennever, 1963). Moreover, calcium plays an important part in the structure of mammalian and bacterial cell membranes and in the structure of bacterial cell walls (Salton, 1964; Keeler and Gray, 1960). Lehninger, Rossi and Greenwalt (1963) have shown in liver cells that calcium and phosphate can form hydroxyapatite in or in association with mitochondrial membranes.

The intercellular calcium phosphate of the plaque is likely to be largely that associated with the carbohydrate-protein which appears to be of salivary origin, mainly from the sub-mandibular glands (Chatterjee, Reddy and Kleinberg, 1973). Cross and Kleinberg (unpublished results) have shown that this

complex of calcium phosphate and carbohydrate-protein upon secretion will precipitate and therefore will likely be present in centrifuged sediment albeit but in lesser amount than in plaque. If so, this would explain the similarity of their release under the different conditions explored.

Effect of Fluoride on Acid-Base Changes at Constant pH

In the constant pH experiments, between pH 5.0 and 8.0, 5 ppm fluoride stimulated acid formation in sediment, plaque and sediment plus hydroxyapatite when each was incubated with glucose. At pH 4.0 and 9.0, opposite effects of fluoride on acid formation with plaque and sediment were observed; hydroxyapatite alone and hydroxyapatite when added to sediment showed similar effects.

The various effects of fluoride can be interpreted as follows. Between pH 5.0 and 8.0, fluoride, in the absence of salivary supernatant (omitted in this study because of its complicating effect on calcium and phosphate changes in the incubation mixtures; Khanna and Kleinberg, 1968) stimulates glycolysis. In the presence of supernatant, fluoride has the opposite effect -- it inhibits glycolysis. Lilienthal in experiments on sediment carried out in bicarbonate buffer at pH 7.0, also found a stimulatory effect with fluoride. This type of result has also been noted in other cellular systems (Carlson and Suttie, 1967). On the other hand, it has been shown that fluoride inhibits glycolysis in pure cultures of streptococci incubated in phosphate buffer at

pH 7.0; (Hamilton 1969b); fluoride also has the same effect on salivary sediment when incubated in the same phosphate buffer. These experiments suggest that fluoride when at low levels is stimulatory; whereas, in phosphate buffer or saliva (which contains phosphate) fluoride is inhibitory. It is therefore conceivable that a phosphate fluoride interaction may be involved.

In the presence of fluoride, the need for smaller amounts of acid and base to keep the pH at 4.0 and 9.0 respectively in both hydroxyapatite suspensions and sediment mixtures containing hydroxyapatite can be attributed to a decreased solubilization in both cases of the hydroxyapatite. Addition of acid at pH 4.0 and base at pH 9.0 replaces the acid and base utilized respectively as hydroxyapatite dissolves (Levinskas and Neuman, 1955). In other words, the effect of fluoride on the acid-base changes would in both cases be through calcium phosphate solubility.

On the other hand, the opposite effects of fluoride at pH 4.0 and 9.0 observed for plaque and sediment may simply be the result of its inhibition of glycolysis. Many studies have shown that below pH 5.0 fluoride inhibits acid formation; at pH 9.0, the effects of fluoride have not been explored. It is interesting to note that fluoride has the same effects in both the presence and the absence of salivary supernatant when the pH is below 5.0; whereas, at neutral pH the effects are opposite.

Both inhibition of acid formation and base formation probably occurred because in both cases the pH rapidly reached levels where fluoride was inhibitory and stimulatory respectively.

Neither the stimulatory effects of fluoride on acid formation at neutral pH nor its inhibitory effects on base formation with urea can be explained by a mechanism involving enolase. The findings of Jenkins (1969) that bacteria grown in a medium containing fluoride store fluoride, and that plaque fluoride (at least in one-day plaque) is associated mainly with the cells rather than the matrix of the plaque suggests that the effect of fluoride is via a membrane mechanism. The observation that bacteria grown in the absence of fluoride are more sensitive to fluoride inhibition than those grown in its presence in consistent with the hypothesis that a limited number of sites for retaining fluoride would be present in the membrane. Thus, saturation of these sites might give the impression of adaptation to fluoride.

Jenkins (1962) has found that the fluoride content of salivary sediment is much lower than that of plaque. His results and those in the present study indicate that acid formation in sediment is more sensitive to fluoride inhibition than in plaque. Besides the higher buffering that occurs in plaque which would inhibit the fluoride effect on acid formation, the possibility

that the plaque has more sites containing fluoride than does sediment and is therefore less sensitive to inhibition by added fluoride is also a possible explanation.

It is tempting to suggest that fluoride becomes attached to the calcium and/or phosphate in membranes (as CaF, fluorophosphate, fluorapatite, or other complex involving protein) in such a way that it alters the release of calcium phosphate into the cellular cytoplasm. The P_i could stimulate glycolysis (Minakami and Yoshikawa, 1956); the Ca^{++} by competing with Mg^{++} might inhibit glycolysis (Lohmann and Meyerhof, 1934). One cannot rule out the possibility that fluoride affects membrane permeability by affecting transport processes and/or configurational changes as suggested by Sandham and Kleinberg (1969). Thus, substrate uptake would be altered and therefore so would the rate of glycolysis (Carlson and Suttie, 1967).

CHAPTER V

THE RELATION OF THE STUDY TO CARIES, CALCULUS FORMATION, AND TOPICAL FLUORIDE THERAPY

It is generally accepted that as the pH of the dental plaque falls, at some point a pH will be reached at which the environment of the tooth will no longer contain sufficient calcium and phosphate to prevent solubilization of the underlying enamel. This pH is called the critical pH. In the absence of plaque the critical pH would be determined for enamel of a particular composition by the composition of the saliva, more particularly its levels of calcium and phosphate. Once plaque forms, however, several things will happen. One, the pH will fall and a lower pH will occur on the enamel surface when fermentable carbohydrate is eaten. Two, the plaque will contain high levels of calcium and phosphate since their deposition is an integral part of the formation of the plaque. Three, plaque fluid rather than saliva will by the fluid in contact with the enamel (Jenkins, 1966).

The present study indicates that a decrease in the pH of the plaque will favour the release of calcium and phosphate. This in turn would increase their levels in the plaque fluid and by mass action retard the loss of mineral from the tooth.

Thus, the plaque calcium phosphate would offset the increased capacity of the plaque to form acid. Since H^+ would be consumed as the calcium and phosphate of the plaque are solubilized, some of the acid formed would be neutralized and this would raise the minimum pH. This in turn would have an indirect effect on the critical pH since it would increase the ion product of calcium phosphate within the plaque, by raising the proportion of the total phosphate present as $HPO_4^=$ and $PO_4^{\equiv}$ (the two phosphate forms pertinent to the solubility of calcium phosphate at physiological pH and to the types of calcium phosphates observed in plaque).

Should the pH subsequently rise and the solubilized calcium and phosphate not have been lost from the plaque, then these ions could participate in the remineralization of the enamel (Koulourides and Reed, 1964) or be transformed into calculus.

The ability of the plaque microflora to rapidly catabolise urea and produce a high pH has been demonstrated both *in vivo* and *in vitro* (Fig. 3 and 4; see also Singer and Kleinberg, 1969). In the absence of dietary carbohydrate, the pH of plaques are generally higher than that of the saliva washing them. Kleinberg and Jenkins (1964) attribute this to the formation of base from the urea which is continually available

in the saliva. Further, Kleinberg and Hall (1969) observed that the pH of the gingival crevice is higher than that of the supra-gingival plaque. Also urea concentrations several times higher in clinically healthy or mildly inflamed gingival crevices than in serum or saliva have been demonstrated (Golub et al, 1971) and may be responsible for the high pH of the crevice. This evidence suggests that the catabolism of urea by the plaque and crevicular microflora produces the pH conditions in the plaque-dentogingival environment that would favour the precipitation of both the plaque calcium and phosphate (Dawes and Jenkins, 1962) and the calcium and phosphate of the gingival crevice fluid (Weinstein et al, 1967). The first would presumably favor supra- and the latter subgingival calculus. Moreover, a higher pH would favour the transformation of any initial deposits that might be amorphous to more crystalline and less soluble forms, most probably hydroxyapatite (Kaufman and Kleinberg, 1970). The suppression of calcium phosphate release during urea catabolism as shown in this study is consistent with the above clinical findings. It is interesting to note that the use of acetohydroxamic acid, a specific urease inhibitor, in rats increased caries incidence and decreased calculus formation (Regolati and Muhlemann, 1971).

Koulourides et al (1964) in an <u>in vitro</u> plaque system found a strong synergism between fluoride, calcium and phosphate

ions in reducing the extent of softening of enamel surfaces. Inhibition of softening was not evident when either fluoride or calcium and phosphate were added. They speculated that fluoride favours the deposition of calcium and phosphate at the sites of demineralization or it may lower the concentrations required to protect the enamel from demineralization.

The considerable suppression of plaque calcium and phosphate release seen in this study with 5 ppm fluoride suggests that less calcium and phosphate would be available for remineralization of an early enamel caries lesion. Moreover, if calcium, phosphate and fluoride are present in solution in the plaque at the same time they could interact and the fluoride precipitate as fluorapatite. As a result a poorly soluble calcium phosphate would be present which would reduce removal of acid (produced by carbohydrate degradation) that would occur if a more soluble calcium phosphate were present and solubilized. Thus, the effect of fluoride in a plaque would have the opposite effect upon the caries process than fluoride in enamel.

That dental plaque has the capacity to concentrate considerable quantities of fluoride is well established (Hardwick and Leach, 1962; Jenkins, 1969). Only a small fraction of the total fluoride in plaque is ionized (Jenkins,

1969; Singer et al, 1970). Hassel et al (1971) using a miniature fluoride electrode with a telemetric recorder showed that following rinsing with a fluoride solution, ionized fluoride disappeared and this occurred more quickly with increasing amounts of interdental plaque.

In view of these observations and those of the present study, it would appear that meticulous removal of plaque (and calculus, which concentrates fluoride to a considerably greater extent than enamel; Hellstrom, 1970) should precede topical fluoride therapy in order that the fluoride ion be made accessible to the enamel and not be incorporated into the plaque where it might decrease the effectiveness of the release of and the buffering by the plaque calcium and phosphate (Stookey and Katz, 1972).

These findings also suggest that a therapeutic agent that raises the plaque calcium phosphate level yet does not hinder its release when the acid attack occurs, should be successful in reducing dental caries. Phosphates such as calcium sucrose phosphates (Smythe, 1971) and phytates may be suited for this purpose since they would favour calcium phosphates of lower Ca/P ratio to be deposited which are also more soluble.

CHAPTER VI

SUMMARY AND CONCLUSIONS

A considerable body of knowledge has accumulated implicating dental plaque in the etiology of both dental caries and periodontal disease. Previous studies have shown that formation of acid during fermentation of dietary carbohydrate by the plaque bacteria results in a decrease in the plaque pH and conditions favourable for enamel dissolution and dental caries. On the other hand, base formation during the metabolism of nitrogenous substrates, particularly urea, results in a rise in the plaque pH and conditions favourable for calculus formation.

High levels of calcium and phosphate, largely in the form of calcium phosphate salts have been found in the dental plaque. Their solubilization under acidic conditions may protect the enamel against caries, whereas, their retention under basic conditions may lead to calculus formation.

The purpose of the first study in this thesis was to obtain an understanding of how the range of pH observed in plaque *in situ* might affect the release or retention of plaque calcium and phosphate. Experiments, therefore, were carried out in which the pH of plaque suspensions was varied in a variety of ways and the release or retention of calcium and phosphate during incubation for several hours at 37°C was

measured. Since salivary sediment contains low levels of calcium and phosphate but yields pH changes similar to plaque when incubated with glucose or urea, experiments were carried out with suspensions of salivary sediment, calcium phosphate and salivary sediment plus added calcium phosphate which enabled quantitative assessment of the effect of solubilization of calcium phosphate on acid-base changes. Adding calcium phosphate to sediment permitted a closer comparison of the interaction between the solubilization and acid-base processes in it and in dental plaque. Hydroxyapatite and dicalciumphosphate were the two forms of calcium phosphate added to sediment since these are the forms of calcium phosphate that have been observed in dental plaque.

In one series of experiments, the pH was varied by incubation of plaque or sediment mixtures with glucose or urea. The pH rose to about 9.0 with urea and fell as low as 4.0 with glucose. In the plaque mixtures incubated with glucose, both calcium and phosphate were progressively released, calcium more rapidly than phosphate. In those mixtures incubated with urea, little or no calcium was released, whereas, release of phosphate was appreciable. The same results were observed with salivary sediment, except that the amounts of calcium and phosphate released from sediment were much less than those observed with

plaque. Addition of synthetic hydroxyapatite to sediment, however, raised the amounts solubilized to levels comparable to those observed with plaque.

In the experiments in which the pH was adjusted and held constant at several levels between 4 and 9, calcium release was inversely related to the pH. The logarithm of the calcium concentration decreased linearly with increase in pH, the slope being about 0.2 for either plaque or sediment and 0.5 for hydroxyapatite, dicalciumphosphate or sediment containing added hydroxyapatite. Release of phosphate showed a similar relationship with pH to that for calcium below pH 6.0 to 8.0 but not above. There, phosphate release increased.

Solubilization of hydroxyapatite and dicalciumphosphate below pH 5.0 required the addition of comparatively large amounts of NaOH to keep the pH constant. Between pH 5.0 and 8.0, however, dissolution required the addition of only small amounts of acid or base; NaOH at acidic, HCl at alkaline pH. HCl was required in much larger amounts at pH 9.0 to offset the effects of solubilization. This was also true for plaque, sediment and sediment containing added hydroxyapatite. Thus, calcium phosphates buffer not only at acidic but also at alkaline pH.

The results were consistent with the view that the plaque

calcium and phosphate would be released during glycolysis and would protect the enamel against dental caries by (i) retarding the loss of calcium phosphate from the enamel (ii) neutralizing some of the acid formed and (iii) providing calcium and phosphate for the remineralization of the enamel surface.

In the second study in this thesis, the effect of fluoride on the interaction between plaque, acid-base formation and calcium phosphate solubilization within the plaque was examined. Experiments were carried out similar in design to those of the first study, except for fluoride being added to the experimental and omitted from the control suspensions. Fluoride (5 ppm) added to sediment or plaque mixtures incubated with glucose inhibited the pH fall. Whereas fluoride inhibition of the fall was slightly evident with plaque, it was clearly evident with sediment. Fluoride also inhibited the rise in pH during the incubations with urea; again, the inhibition was slightly evident in the plaque and clearly evident in the sediment mixtures. Addition of hydroxyapatite resulted in fluoride actually stimulating the pH fall slightly in the mixtures with glucose but had no effect in the mixtures with urea.

Fluoride suppressed the release of calcium and phosphate in the plaque or sediment mixtures incubated with glucose and the release of phosphate in the plaque mixtures incubated with

urea. Fluoride had no effect on sediment mixtures similarly incubated with urea. The constant pH experiments showed that fluoride inhibited calcium and phosphate release over the whole pH range examined but the pattern of release was not affected. Interestingly fluoride stimulated acid formation from glucose above but inhibited acid formation below pH 5.0.

Because of the dual effect of fluoride on acid formation and calcium phosphate solubilization, and the fact that acid formation and calcium phosphate solubilization have opposite effects on the pH, it was evident from the results that any effects on fluoride on the pH of the dental plaque would not be readily apparent. This was evident from the fact that adding either hydroxyapatite or fluoride to sediment inhibited the pH fall, whereas, the addition of both hydroxyapatite and fluoride to sediment and only fluoride to plaque had small effects on the pH. Interestingly, fluoride stimulated the pH fall slightly with sediment plus hydroxyapatite but inhibited the fall slightly with plaque. The calcium and phosphate results indicated that fluoride was inhibitory of their release in all cases.

It was pointed out that suppression of the release of plaque calcium and phosphate could lead to decreased protection of the enamel since under acidic conditions the plaque calcium

and phosphate that would be solubilized to protect the tooth from caries would be reduced.

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