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 form-

ation when urea is substituted as substrate (Stephan, 1943;

Frostell, 1959; 1960; Singer and Kleinberg, 1969). Rapid form-

ation 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, diffi-

culties 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 dif-

ferences 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 pre-

dominant 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 pyrophos-

phate results when the diet is supplemented with orthophosphate.

If the salivary pyrophosphate level is also increased, then

orthophosphate supplements might lead to decreased calculus for-

mation.

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 sarco-

plasmic 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,