1973

Chemical Kinetics, Density And Electron-Microscopy Of Enamel Surface Reactions.

Avila Manuel Gonzalez
University of Alabama at Birmingham

CHEMICAL KINETICS, DENSITY AND ELECTRON MICROSCOPY OF ENAMEL SURFACE REACTIONS

by

MANUEL GONZALEZ AVILA

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in The Graduate School of the University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1973

ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. Fred F. Feagin, my faculty advisor, for his interest, friendship and guidance during my graduate program.

I also wish to express my gratitude to Dr. Sam Barker for his encouragement and his direct interest in my research.

I extend my appreciation to Drs. Edwin Bradley, Charles Bugg, Marshall Hartley, Ted Koulourides and Ward Pigman for serving as members of my graduate committee.

Support was given to this work from research grants of the Veterans Administration and the Institute of Dental Research of the School of Dentistry, University of Alabama in Birmingham, through Dr. Fred F. Feagin.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	v
LIST OF ILLUSTRATIONS	vi
CHAPTER	
I. INTRODUCTION	1
Nature of the Mineral of Calcified Tissues	1
Mineralization of the Enamel Surface	3
II. FACTORS AFFECTING MINERAL ACCRETIONS IN THE ENAMEL-SOLUTION INTERFACE	7
Introduction	7
Materials and Methods	8
Results	15
Discussion	44
Summary	49
III. DENSITY AND X-RAY DIFFRACTION STUDIES OF ENAMEL SURFACE REACTIONS	50
Introduction	50
Materials and Methods	51
Results	59
Discussion	68

Table of Contents (Continued)

	Page
Summary	71
IV. ELECTRON MICROSCOPY OF THE ENAMEL SURFACE BEFORE AND AFTER MINERALIZATION REACTIONS	73
Introduction	73
Materials and Methods	74
Results	79
Discussion	99
Summary	102
V. CONCLUSIONS	104
BIBLIOGRAPHY	107

LIST OF TABLES

Table		Page
1.	Coefficients of Determination for Fit of the Assumed Models for the Variables lnCa, lnP, lnH, and lnF.....	31
2.	Means and Standard Errors of the Variables $lnCa_o$, Ca Rate, lnP_o, P Rate, H_1, H Rate, lnF_1 and F Rate by pH and Fluoride Level.....	34
3.	Analysis of Variance of the Effect of Fluoride and pH on the Variables $lnCa_o$, Ca Rate, lnP_o, P Rate, H_1, H Rate, lnF_1, and F Rate.....	36
4.	Density of Powder Scraped from Untreated and Experimentally Altered Enamel Surfaces and Synthetic Apatite.....	62
5.	Reactions of Calcium and Phosphate at Surfaces of Acid-Etched Enamel Immersed in Metastable Solutions of Constant pH 7.0.....	80

LIST OF ILLUSTRATIONS

Figure		Page
1.	System Used to Maintain Constant pH and pF in a Recirculating Solution During Interfacial Reactions with Enamel Minerals	10
2.	Kinetic Approach to Chemical Equilibrium of the Enamel-Solution Interface	17
3.	Effect of 0.05 mM [F] on the Kinetic Reactions of the Enamel-Solution Interface	22
4.	Effect of pH on the Kinetic Reactions of the Enamel-Solution Interface	24
5.	Kinetics of Fluoride Changes at the Enamel-Solution Interface	27
6.	Effect of Flow Rate on the Reactions at the Enamel-Solution Interface	30
7.	Reaction Rates of Calcium as a Function of pH with Upper and Lower 95 Percent Confidence Limits	38
8.	Reaction Rates of Phosphate as a Function of pH with Upper and Lower 95 Percent Confidence Limits	41
9.	Reaction Rate of Fluoride as a Function of pH. Means and Standard Errors	43
10.	Apparatus Used for the Preparation of Density Gradient Columns	55
11.	Density Gradient Column Containing Sinkers of Known Density and Calibration Graph Showing Density Range and Slope	58
12.	Changes of Calcium and Phosphate Concentrations During Mineral Accretion at the Enamel-Solution Interface	61

List of Illustrations (Continued)

Figure		Page
13.	Density of the Powder Scraped from Untreated and Experimentally Altered Enamel Surfaces, and Synthetic Apatites	64
14.	X-ray Powder Diffraction Patterns of Enamel Surface Density Fractions	67
15.	Diagram of the Preparation of Enamel for Ultramicrotomy	78
16.	Transmission Electron Micrograph of Lightly Abraded Labial Enamel Surface of Bovine Incisors	83
17.	Transmission Electron Micrograph of the Labial Surface of Enamel After 14 Hour Immersion in a Solution of 2 mM Sodium Acetate Buffer at pH 5.0	83
18.	Transmission Electron Micrograph of the Labial Surface of Enamel After 14 Hour Immersion in a Solution of 2 mM Sodium Acetate Buffer at pH 5.0	85
19.	Transmission Electron Micrograph of the Labial Surface of Acid-Etched Enamel After 4 Hour Reaction with a Metastable Calcifying Solution	85
20.	Transmission Electron Micrograph of the Labial Surface of Acid-Etched Enamel After 8 Hour Reaction with a Metastable Calcifying Solution	88
21.	Transmission Electron Micrograph of the Labial Surface of Acid-Etched Enamel After 4 Day Reaction with a Metastable Calcifying Solution	88
22.	Transmission Electron Micrograph of the Mineral Deposited on the Surfaces of Enamel After Spontaneous Precipitation of a Calcium Phosphate Solution	90
23.	Transmission Electron Micrograph of Untreated Bovine Enamel	93

List of Illustrations (Continued)

Figure		Page
24.	Transmission Electron Micrograph of Dental Enamel Reacted with a Solution of 2 mM Sodium Acetate Buffer at pH 5.0	93
25.	Transmission Electron Micrograph of the Mineral Formed on the Enamel Surface After 4 Days of Reaction with a Metastable Calcifying Solution	93
26.	Transmission Electron Micrograph of the Mineral Deposited on the Surfaces of Enamel After Spontaneous Precipitation of a Calcium Phosphate Solution	93
27.	Electron Diffraction Patterns of Selected Areas of the Enamel Surfaces	95
28.	Scanning Electron Micrograph of a Lightly Abraded Surface of Bovine Enamel	98
29.	Scanning Electron Micrograph of Enamel Surface Reacted with a Solution of 2 mM Sodium Acetate Buffer at pH 5.0	98
30.	Scanning Electron Micrograph of Acid-Etched Enamel Surface After 8 Hour Reaction with a Metastable Calcifying Solution	98
31.	Scanning Electron Micrograph of Acid-Etched Enamel Surface After 48 Hour Immersion in a Metastable Calcifying Solution	98

CHAPTER I

INTRODUCTION

Hardness, the most distinct of the properties of calcified tissues can be directly attributed to the structural relations between inorganic salts and organic matrix (Eanes and Posner, 1970). Less obvious, but significant, is the high reactivity of these tissues, especially the inorganic phase.

Nature of the Mineral of Calcified Tissues

The chemical composition of the mineral of bones and teeth varies greatly, not only from individual to individual, but also among different areas of the same organ. About 50 to 70 percent of bone and dentin, and about 96 percent of dental enamel consist of an inorganic material with chemical composition and stoichiometry similar to ideal hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. Because of its small amount of impurities present in an unknown structural form, the inorganic phase of mammalian mineralized tissues is considered an "apatite-like" mineral. Chemical analyses show that adult human bone contains about 22.5 percent calcium and 10.3 percent phosphate; human dental enamel contains about 35.9 percent calcium and 17.0 percent phosphate on a dry, fat free weight basis (Zipkin, 1970).

Early crystallographers (de Jong, 1926) first observed the close similarity between the X-ray diffraction patterns of bone and apatite. Since then, other structural studies have shown that the mineral of hard tissues has an apatitic lattice arrangement (Bale, 1940). Besides X-ray diffraction, such studies have included electron diffraction (Barker, et al., 1970), neutron diffraction (Young and Spooner, 1969), and infrared spectroscopy (Le Geros, et al., 1968). The crystalline structures of pure fluorapatite and hydroxyapatite were first worked out by Naráy-Szabó (1930) and Posner, Perloff and Diorio (1958), respectively.

Extensive review of the literature indicates that the current knowledge of bone mineral chemistry has been obtained from experiments involving precipitation of apatite from unstable supersaturated solutions. Although the precipitates obtained are similar to bone mineral in some respects, they exhibit non-reproducible chemical and physical characteristics. However, studies of this type may be necessary to understand the behavior of particular families of calcium phosphate minerals.

Recent investigators have indicated the possibility of the presence of a second calcium phosphate structure in significant amounts in recently formed mineral of hard tissues (Molnar, 1959). This structure, commonly referred to as "amorphous" calcium phosphate, does not produce detectable crystalline organization when studied with X-ray diffraction. It has been suggested that noncrystalline calcium phosphate is a precursor phase in the formation of hydroxyapatite (Eanes and Posner, 1970).

Mineralization of the Enamel Surface

Early investigations of the enamel surface indicated that surface enamel was more resistant to acid dissolution than inner enamel (Miller, 1905), but it was not until 1912 (Head, 1912) that indications of dynamic interactions between dental enamel surface and environmental solutions were provided. It was shown by comparison of microindenter penetrations that acid-etched enamel surfaces recovered their hardness when exposed to saliva (Head, 1912). Later investigations demonstrated conclusively that dental enamel, far from being inactive, was a highly reactive substance and incorporated into its mass detectable amounts of radioisotopes (Armstrong, 1955). The isotopes were observed more concentrated in the outer surface of enamel (Sognnaes, 1955).

Microhardness studies have indicated that acid-etched enamel rehardens when immersed in metastable* solutions of calcium phosphate (Koulourides, Cueto and Pigman, 1961). A higher rate of enamel hardness recovery was found when fluoride was added to those solutions or to saliva (Koulourides, Feagin and Pigman, 1965). The relation between the chemical events of demineralization and remineralization and the physical softening and rehardening was demonstrated by Feagin, Koulourides and Pigman (1969).

---

*The term "metastable" refers to solution concentrations of calcium and phosphate supersaturated with respect to apatite but not saturated to the extent of spontaneous precipitation.

Recent investigations demonstrate the simultaneous kinetic reactions of calcium, phosphate, fluoride and hydrogen ions at the interface between the enamel surface and solutions of defined chemical composition (Feagin, Gonzalez and Jeansonne, 1972). It has been suggested that the changes observed during reactions between apatitic minerals and dilute calcifying solutions are due to recrystallization (Neuman and Neuman, 1958), or to crystal growth (Nancollas and Mohan, 1970). Attempts have been made to study the products formed on enamel by those reactions using optical microscopy and microradiography. Johanson (1965) indicated that there is an increase in radiodense substances and an observable change by polarized light microscopy, after an acid-etched surface of enamel was exposed to a mineralizing solution. Equivalent results have been reported in carious enamel using microradiography (Silverstone and Poole, 1969). These findings have been interpreted as indications of mineral growth at the enamel surface.

From the point of view of electron microscopy, enamel demineralization consists of a diffuse destruction of apatite crystals (Frank, 1967; Lenz, 1967). Intracrystal destruction in the form of a central electronlucent area has also been reported (Johansen, 1963; Scott, Simmelink and Nygaard, 1971). The ultrastructural appearance of the mineral accretions on etched surfaces of enamel has been described as "finely grained amorphous precipitate" by Muhlemann, Lenz, and Rossinsky (1964). Their method involved the use of solutions containing calcium, phosphate, and a relatively large

concentration of carbonate. The suggestion has been made that dental enamel remineralizes in vivo not through the surface growth of pre-existing crystals, but through the formation of new apatite crystals recognized by their size and orientation (Lenz, 1967). No attempt has been made to relate the changes observed in the electron microscope with simultaneous changes of the environmental solution or to characterize the minerals deposited at the enamel surface.

Enamel density has often been estimated by indirect procedures such as quantitative microradiography (Angmar, Cärlstrom and Glas, 1963). In 1939, Manly and Hodge isolated enamel fractions that ranged in density from 2.89 to 3.00 g/cm^3 but gave no indication about the original position of the density fractions in the tooth. Human enamel gradually decreases in density from the surface toward the dentin (Cocklica and Brudevold, 1966; Weidmann, Weatherell and Hamm, 1967). This density gradient possibly correlates with different proportions of minerals and organic substances along the enamel thickness (Baud and Lobjoie, 1966). Currently accepted values of human enamel density range from about 2.84 to 3.00 g/cm^3 (Weidmann, et al., 1967).

Atomic scale studies of the enamel structure have been mostly made with X-ray diffraction methods since Bale (1940) first showed the crystalline structure of the enamel mineral. Neutron diffraction (Young and Spooner, 1969) and electron diffraction (Frazier, 1970) have also been used with compatible results. Generally,

crystallographic studies of adult tooth enamel indicate that enamel mineral is a highly crystallized hydroxyapatite-like mineral.

The biologic fluid at the enamel surface in vivo is continuously variable and so complex that it has defied even speculative description. The fact that regardless of the environment, reactions of the enamel mineral must ultimately occur at the surface of the mineral has received little recognition. Much emphasis in dental caries research should be given to the surface reactions of dental enamel since the surface is the initial site of destruction or defense against dental caries and other pathologic processes.

The purpose of this work is to provide experimental observations of the chemical and physical nature of the minerals formed in or around the surfaces of tooth enamel. Specifically, our primary objective is to critically evaluate the density, simple X-ray diffraction classification and electron microscopic appearance of minerals formed at the interface between the enamel mineral and stable solutions containing dilute organic acid or calcium and phosphate ions after first having obtained information of the chemical kinetics of the enamel surface reactions. Factual knowledge of the dynamic reactions between the tooth mineral and a given environmental solution is at present inadequate. Such knowledge is an absolute requirement to the understanding of the interactions of dental enamel with oral fluids and to determine scientifically the direction of future dental caries research.

CHAPTER II

FACTORS AFFECTING MINERAL ACCRETIONS IN THE ENAMEL-SOLUTION INTERFACE

Introduction

Soon after eruption of the tooth, the exposed surface is no longer enamel tissue mineral alone, but, in addition, it has the products of interaction between the enamel mineral, saliva, food debris, and microorganisms (Dirkes, 1966). These products include deposits of apatite-like calcium phosphate minerals, which may react with weak acids to prevent enamel demineralization (Von der Fehn, 1964), or may be involved in remineralization of demineralized surfaces (Koulourides, 1968). Thus, the potential to promote the accretion of interfacial minerals of similar composition and chemical reactivity as the enamel mineral may provide a rational means to effectively prevent dental caries.

Mineral accretions have been demonstrated in vitro in lightly acid-etched surfaces of enamel, as well as in the natural tooth surface (Silverstone and Johnson, 1971), following immersion in saliva, serum, and simple solutions containing small nonprecipitating concentrations of calcium and phosphate (Feagin, Walker and Pigman, 1969). Fluoride ion concentrations of 0.025 to 0.5 mM potentiate

(one-half maximum rate at 0.05 mM) the accretion of calcium phosphate in the enamel-solution interface (Thomas and Feagin, 1973).

The purpose of this work was to study factors affecting dynamic reactions of calcium, phosphate, fluoride, and hydrogen ions in the interface between surfaces of enamel and solutions of known compositions (Feagin, Gonzalez and Jeansonne, 1972). Our objective was to investigate the reactivity of the enamel-solution interface and determine conditions that could possibly favor the formation of mineral accretions of desirable properties in the in situ enamel surface.

Materials and Methods

Preparation of the Enamel Surfaces.

Bovine labial enamel surfaces of 1.00 cm^2 were sectioned under water spray in a thin sectioning machine* equipped with a diamond-impregnated steel blade. The enamel surfaces were mounted on separate plastic slabs with dental sticky wax in the manner indicated in Fig. 1. The labial surface of the enamel was mildly abraded with fine grit silicon carbide paper and, then, polished with wet pumice powder and a dental prophylaxis rubber cup. Twelve cm^2 of enamel surface so treated were used in each experiment, unless otherwise specified in the individual sections of this manuscript.

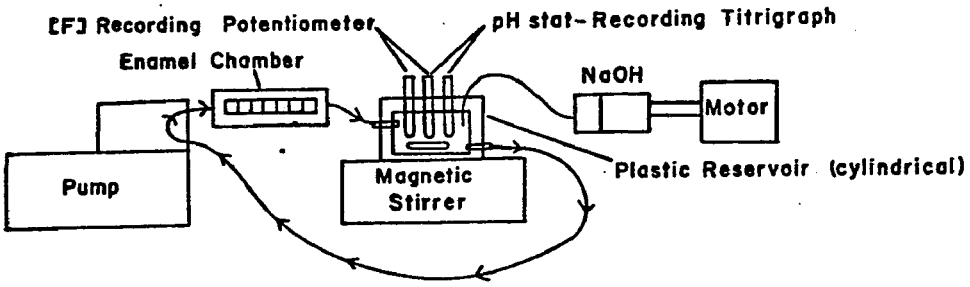
---

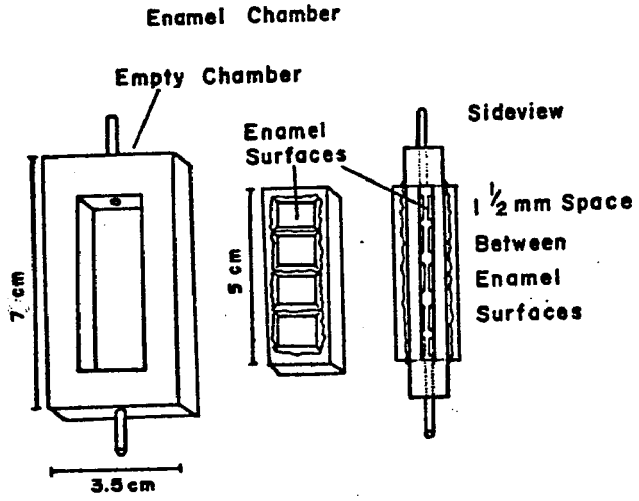
*Gillins-Hamco

Figure 1.

System Used to Maintain Constant pH and pF in a Recirculating Solution During Interfacial Reactions with Enamel Minerals.

The system consisted of a 12 ml capacity reservoir, a polystatic pump and tubing to flow solution to and from the reservoir through the enamel chambers, and hydrogen ion, fluoride ion and double lead reference electrodes sealed into the solution reservoir.


[F] Recording Potentiometer
pH stat-Recording Titrigraph
Enamel Chamber
NaOH
Motor
Pump
Magnetic Stirrer
Plastic Reservoir (cylindrical)


Enamel Chamber
Empty Chamber
Enamel Surfaces
Sideview
1 ½ mm Space Between Enamel Surfaces
7 cm
5 cm
3.5 cm

Stock and Experimental Solutions.

The following stock solutions were prepared from reagent grade chemicals:

1) 1.0 M sodium acetate buffer at pH 5.0
2) 0.05 M [Ca], Ca/P ratio of 1.67, pH 1.5
3) 0.0125 M NaF
4) 0.05 M NaOH
5) 0.05 M HCl, and
6) 1.00 M NaCl.

Volumes of the stock solutions were pipetted into deionized water and diluted close to final concentration. The solutions were brought to final volume after adjustment of pH with NaOH or HCl.

The experimental acid-etching solution was prepared from the sodium acetate buffer stock solution and had a final concentration of 2 mM.

The experimental calcifying solution contained 1.5 mM [Ca], 0.9 mM [P], 0.05 mM [F] and 150 mM [NaCl] at pH 7.0 unless otherwise specified. Titration of the calcifying solutions was done slowly to eliminate the possibility of precipitation of calcium phosphate caused by the sudden addition of high concentration of base.

Procedure for the Study of Chemical Kinetics.

Twelve cm^2 of polished labial enamel were immersed in 2000 ml of a solution of 2 mM sodium acetate buffer at pH 5.0 for 14 hours

at 37°C, and then rinsed in neutral pH distilled water containing 150 mM NaCl for 3 hours; this was done to dilute residual acid of the mineral surface. The acid-etched enamel surfaces were exposed to 12 ml of the calcium phosphate solution in a system that allowed recirculation of the solution, withdrawal of microsamples and potentiometric control of pH and fluoride ion activity (pF). Six of the one cm^2 etched surfaces of enamel were assembled into a plastic chamber of about 1.5 ml volume capacity. One or two of the chambers containing the enamel surfaces were connected in parallel by polyethylene tubing to a pump so that the calcifying solution circulated through a circuit composed of enamel chamber(s), mixing reservoir and a pump (Fig. 1). Before the immersion of the enamel surfaces in the mineralizing solution, the system was operated until steady state was reached. During experimentation, the pH and pF of the solution were continuously monitored and controlled at the desired level by microliter additions of the respective stock solutions. As shown in figure 1, a fluoride ion electrode, a pH electrode and a common calomel reference electrode were sealed into the mixing reservoir. Maintenance of pH and pF was accomplished by the use of potentiometric controlled pumps to keep the electrical potential of each electrode at a constant millivoltage (pH- and pF-stats*). The amount of fluoride ions consumed and hydrogen ions

---

*Orion Research, Inc.: 801 Digital pH/mv meter; 872 Digital Controller. Radiometer TTT 2 Titrator and SBR 3c Titrograph.

produced by the interfacial reactions were measured as the volume of the respective stock solution (0.0125 M NaF and 0.05 M NaOH) required to maintain the fluoride ion activity and the pH of the solution.

Calcium and phosphate concentrations were measured at the time of initial contact between enamel and solution. Withdrawal of duplicate 50 µl samples for calcium analysis and duplicate 50 µl samples for phosphate analysis were made at half hour intervals during the experiment. The experiment was not interrupted by the sampling procedure. Calcium concentration was determined by the method of Sarkar and Chauhan (1967). Phosphate concentration was determined with the method of Chen, Toribara and Warner (1956).

The data of each experiment, i.e., the calcium and phosphate concentrations in solutions and volumes of reagent stock solutions added to maintain pH and pF were corrected for volume alterations at each time of analysis.

Twelve surfaces of labial enamel of bovine incisor teeth were used in each replicate of the experiments. Each of the 8 pH levels studied was replicated 6 times in experiments involving the use of fluoride. Similar experiments were made without fluoride. Duplicate samples for calcium and phosphate analyses were drawn from the experimental solution at half hour intervals during 3 hour reactions. Hydrogen and fluoride ion additions were measured directly from the syringes of the potentiometric pumps as the added volumes of the corresponding solutions.

The reaction rates were approximated by the equation

$$\frac{dY_t}{d_t} = \beta(t)Y_t, \qquad (1)$$

where Y_t is the variable of interest at time t. It was assumed that the rate of change of Y_t was proportional to concentration Y_t, with a rate function $\beta(t)$ possibly changing with time. For calcium ion and phosphate ion reactions we took

$$\beta(t) = \beta, \qquad (2)$$

i.e., the rate of change of concentration of calcium or phosphate in solution at time t is proportional to the concentration of calcium or phosphate respectively. Substituting equation (2) into (1) and solving yields

$$\ln Y_t = \ln Y_o + \beta t, \qquad (3)$$

where Y_t is [Ca] or [P] at time t, Y_o is initial [Ca] or [P] and β is Ca rate or P rate.

For hydrogen and fluoride ion reactions, we took

$$\beta(t) = \beta t^{-1}, \qquad (4)$$

i.e., rate of change of the addition of hydrogen and fluoride ions at time t is proportional to the amounts previously added in the interval (o,t) and decreases with time.

Substituting (4) into (1) and solving yields

$$\ln Y_t = \ln Y_1 + \beta \ln t, \qquad (5)$$

so that, hydrogen and fluoride ions relate to time by the power function.

Estimation of the unknown parameters of equations (3) and (5) was by the method of least squares for each of the replicates. Coefficients of determination were obtained in order to determine the proportion of variation of the variables explained by the model and the proportion given by lack of fit of the model.

Results

Effect of 25 μM or 50 μM Fluoride Ion Concentrations on Reactions of the Enamel-Solution Interface in Solutions of Constant pH 7.0.

Figure 2A shows the time dependent changes of calcium and phosphate concentrations in solutions containing no fluoride, during the enamel-solution interfacial reactions. Figure 2B shows the changes of calcium and phosphate in solutions containing 0.05 mM fluoride. Figure 2C shows the amounts of fluoride and hydroxyl ions added to the solutions to maintain constant 0.05 mM fluoride ion concentration and pH 7.0 during reactions. The solutions contained 1.5 mM calcium and 0.9 mM phosphate at the initial time of contact of enamel and solution. The figure ordinates show the change of calcium and phosphate concentration and the additions of hydroxyl ion and fluoride ion from reagent stock solutions.

The rates of the interfacial reactions were faster during the initial 3 to 6 hours and, thereafter, approached chemical equilibrium as the calcium and phosphate concentrations decreased 60 to 70%. The

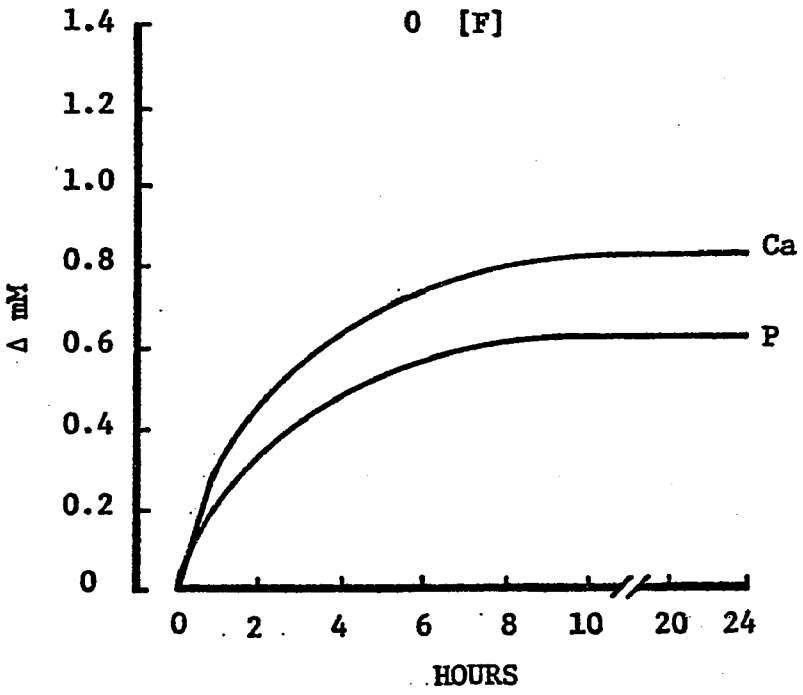
Figure 2.

Kinetic Approach to Chemical Equilibrium of the Enamel-Solution Interface.

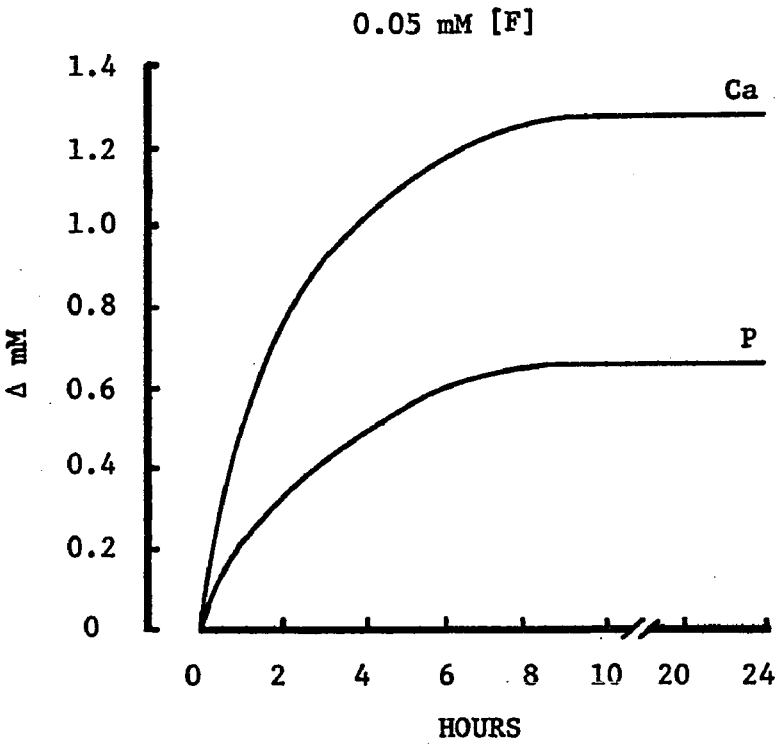
Twelve cm^2 enamel were exposed to 12 ml of solution initially containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0 or 0.05 mM [F]. Solution decreases in Ca and P are indicated, as well as H^+ additions to maintain constant pH 7.0, and F^- additions to maintain constant $[F^-]$.

A. Changes of calcium and phosphate concentration in the absence of fluoride.
B. Changes of calcium and phosphate concentration in the presence of 0.05 mM fluoride.
C. Amounts of hydrogen ions added to maintain constant pH in the presence or absence of fluoride and amount of fluoride ions added to maintain constant 0.05 mM [F].

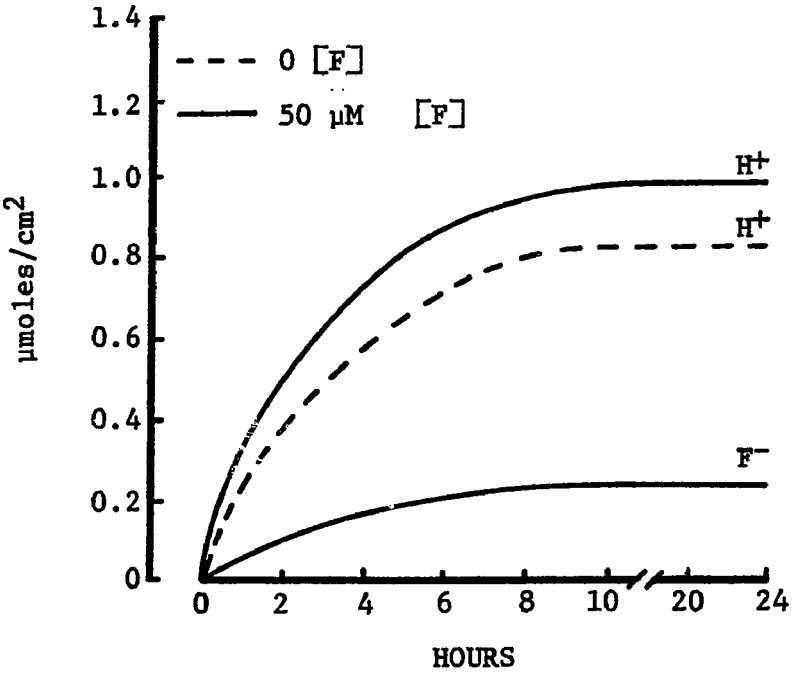
A.



B.



C.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
µmoles/cm²
0 [F]
50 µM [F]
H+
H+
F⁻
0
2
4
6
8
10
20
24
HOURS

rates of the reactions were greater in solutions containing 0.05 mM fluoride than in solutions without fluoride. Reactions were essentially complete at about 8 hours.

Figure 3 shows the rates of calcium and phosphate reactions of the enamel-solution interface during the first 3 hours as percentages of the calcium and phosphate concentrations of the solution at the initial time of enamel-solution interface. The results were obtained in experiments additional to, and under the same conditions (1.5 mM calcium and 0.9 mM phosphate, i.e., 1.67 Ca/P) as those shown in figure 2. The coefficients of linear correlation were between 0.7 and 0.9 for the regression lines. The rates of the calcium and phosphate reactions were greater in the interface in the solutions with 0.05 mM fluoride ion than in the solutions without fluoride. The calcium and phosphate reaction lines were practically coincident in the solutions containing fluoride. In contrast, the rates of the phosphate reactions were greater than the rates of calcium reactions in the solutions without fluoride.

Effect of pH on Reactions of the Enamel-Solution Interface.

Figure 4 shows phosphate concentrations in solutions of constant pH 4.6, 5.0, 5.4, 5.8, 6.2, 6.6, 7.0 or 7.4 during reactions of the enamel-solution interface. The solutions initially contained 1.5 mM calcium and 0.9 mM phosphate and 0 or 0.05 mM fluoride. The ratio of enamel surface to solution volume was 12 cm^2/12 ml. The

Figure 3.

Effect of 0.05 mM [F] on the Kinetic Reactions of the Enamel-Solution Interface.

Percentage reactions of solution Ca and P are shown during exposure of 12 cm^2 enamel to 12 ml of solution initially containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl], and 0 or 0.05 mM [F]. Fluoride ion concentration was maintained constant, as was pH 7.0.

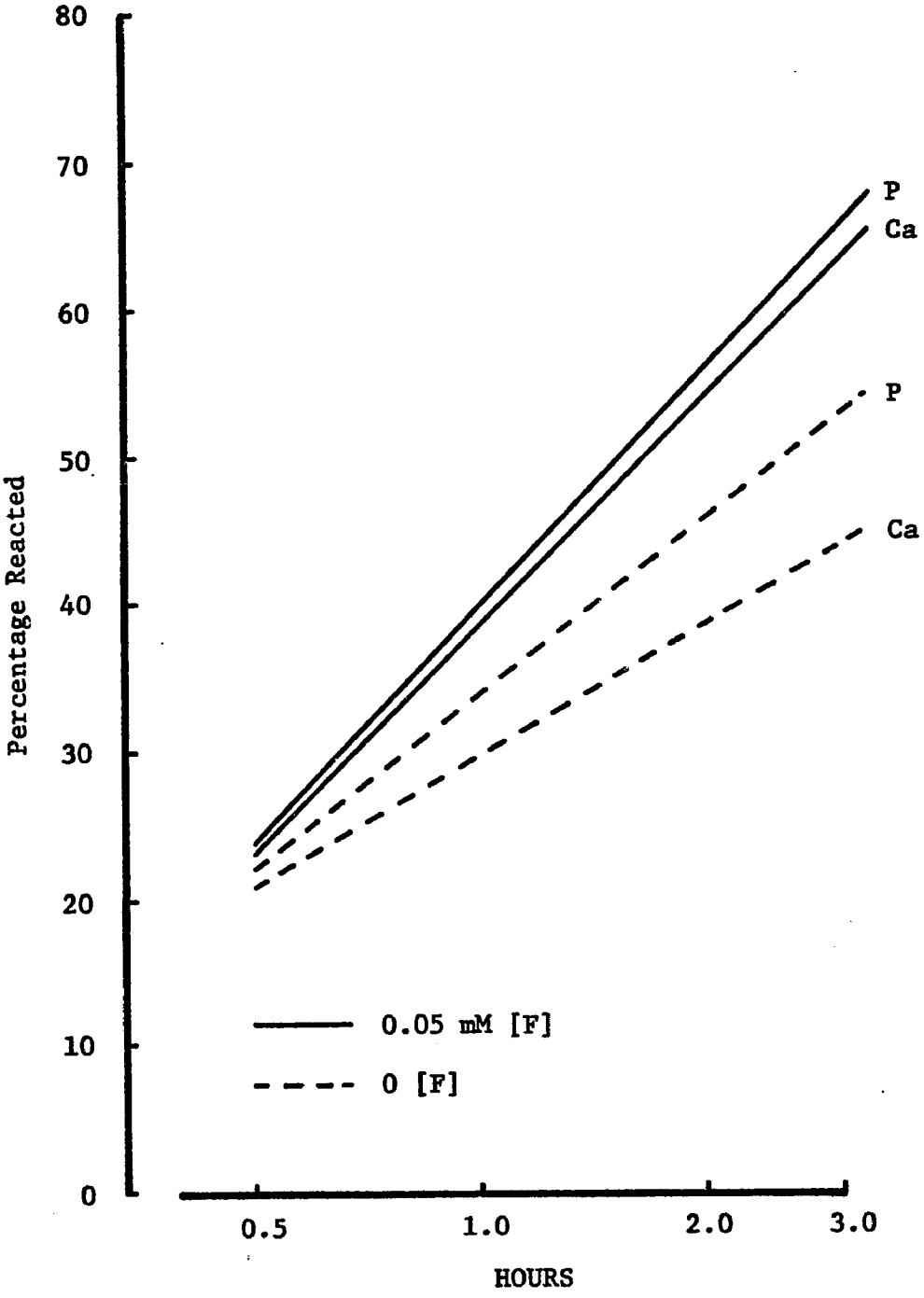
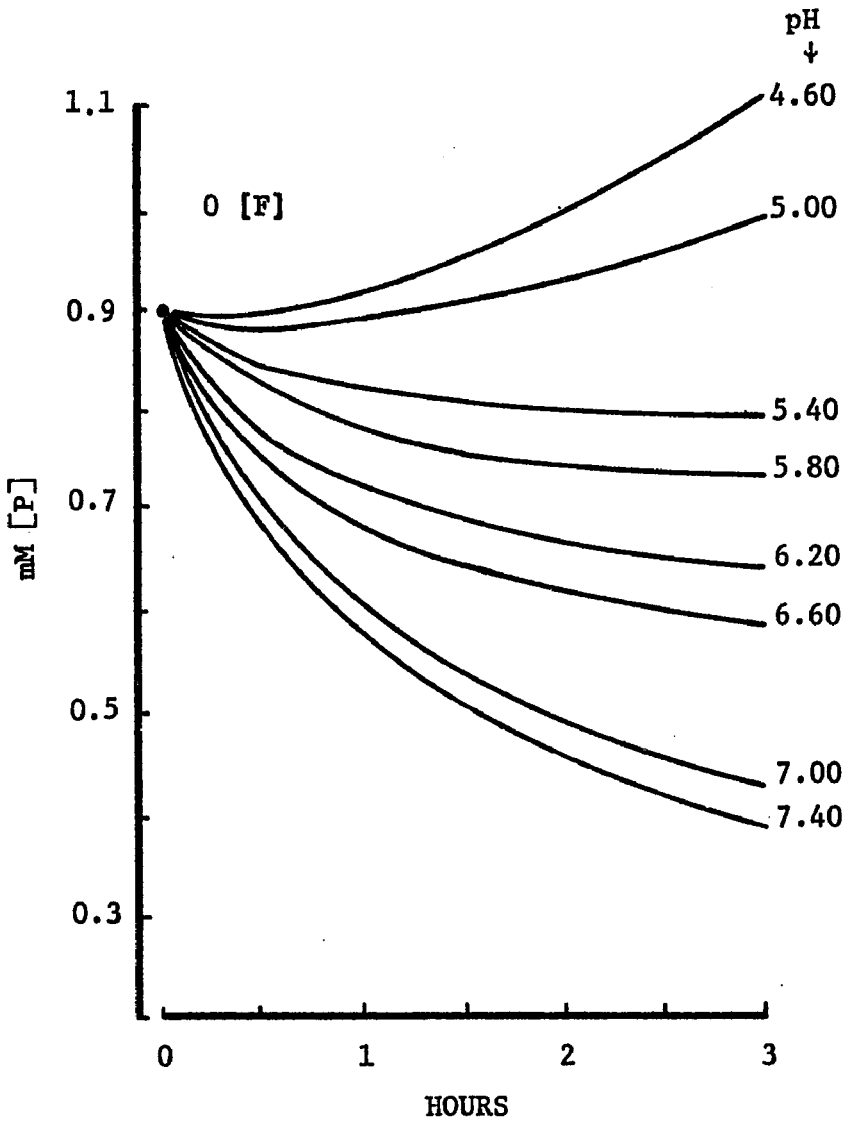
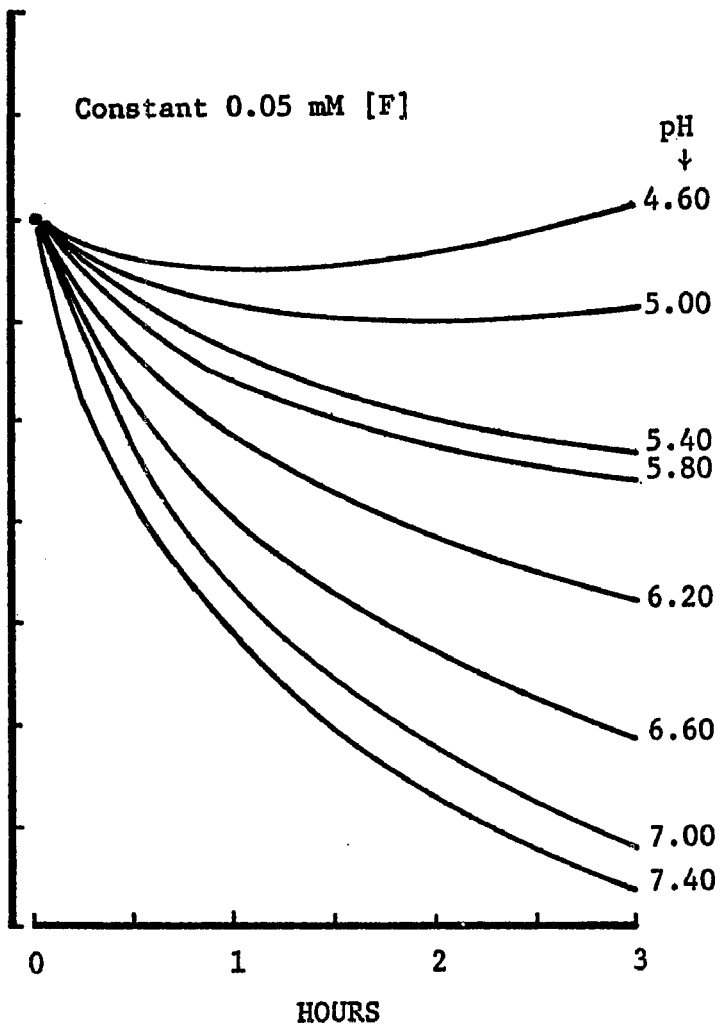

80
70
60
50
40
30
20
10
0
Percentage Reacted
P
Ca
P
Ca
0.05 mM [F]
0 [F]
0.5
1.0
2.0
3.0
HOURS

Figure 4.

Effect of pH on the Kinetic Reactions of the Enamel-Solution Interface.

Twelve cm^2 of enamel were exposed to 12 ml of solution containing initially 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl], and 0 or constant 0.05 mM [F] at constant pH 4.60, 5.00, 5.40, 5.80, 6.20, 6.60, 7.00 or 7.40. Phosphate concentration changes with or without fluoride are indicated.


pH
4.60
5.00
5.40
5.80
6.20
6.60
7.00
7.40
0 [F]
1.1
0.9
0.7
0.5
0.3
mM [P]
0
1
2
3
HOURS


Constant 0.05 mM [F]
pH
4.60
5.00
5.40
5.80
6.20
6.60
7.00
7.40
0
1
2
3
HOURS

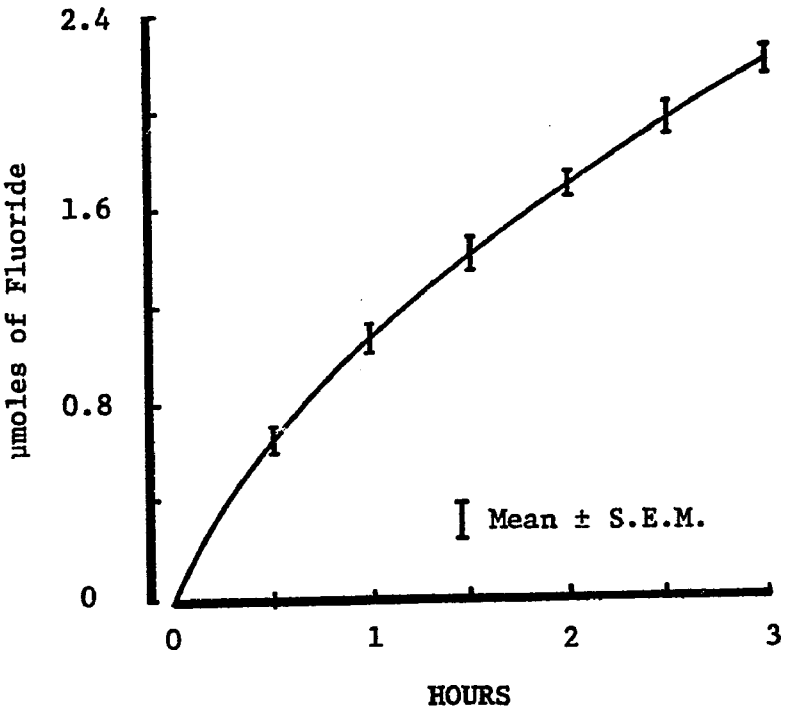
fluoride ion concentration was maintained constant during reactions at each pH. Curves of similar shape were obtained for the concurrent reactions of calcium as for those illustrated for the phosphate reactions. The phosphate concentrations increased at pH 4.6 and 5.0 in the solutions without fluoride, whereas the phosphate concentrations were practically unchanged in solutions with 0.05 mM fluoride at the same pH's. The phosphate concentrations decreased as a family of curves as the pH in solutions were progressively increased above pH 5.0.

Figure 5 shows the rates of fluoride uptake in the enamel-solution interface during the reactions of calcium, phosphate and hydrogen ions illustrated in figure 4. The curve in figure 5 represents the mean and standard error of 48 separate experiments, i.e., 6 experiments at each constant pH of 4.6, 5.0, 5.4, 5.8, 6.2, 6.6, 7.0 and 7.4. The rates of uptake of fluoride were affected neither by the change of pH of the solutions nor by the reaction rates of calcium, phosphate, or hydrogen ions. The fluoride reaction curve shows the additions of the stock fluoride solution, as μmoles fluoride, to maintain constant electrode potentials equivalent to the potential of standard 0.05 mM sodium fluoride solutions of the same pH as the experimental solutions.

Figure 5.

Kinetics of Fluoride Changes at the Enamel-Solution Interface.

Twelve cm^2 of enamel were exposed to 12 ml of solution containing initially 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl], and constant 0.05 mM [F] at pH 4.60, 5.00, 5.40, 5.80, 6.20, 6.60, 7.00 or 7.40.


2.4
1.6
0.8
0
µmoles of Fluoride
Mean ± S.E.M.
0
1
2
3
HOURS

Effects of the Flow Rate on the Reactions at the Enamel-Solution Interface.

Figure 6 shows the relationships between solution flow rate and the reactions of calcium, phosphate, hydrogen and fluoride ions in the enamel-solution interface. The solutions contained 1.3 mM calcium and 0.9 mM phosphate at the initial time of interfacial reaction. The ratio of enamel surface to solution volume was 12 cm^2/12 ml. The values of the reactions at the 2 hour time of enamel-solution contact were plotted versus the flow rate of the solutions at the interface. The rates of the reactions sharply increased with increased flow rates from 0.5 to 4 ml/min. The rates of the calcium, phosphate, and fluoride ion reactions were practically constant at flow rates of 4 to 10 ml/min. However, the rates of solution flow through the mixing reservoir were too rapid at flow rates greater than 6 ml/min. for accurate pH control.

Table 1 shows the coefficients of determination of the term $\ln Y_t$ of equations 3 and 5 for the variables calcium, phosphate, hydrogen and fluoride concentrations. The coefficients that showed lack of fit to the mathematical model at the 0.05 probability value appear marked with an asterisk. Only the variables lnH at pH 5.80 and lnCa at pH 5.40 showed significant lack of fit with more than 5 percent of the variation not explained by the mathematical model. Other values of the variables lnH and lnF showed significant (0.05 probability level) lack of fit, but, since the proportion of unexplained variation is less than 5 percent, it can be assumed that

Figure 6.

Effect of Flow Rate on the Reactions at the Enamel-Solution Interface.

Twelve cm^2 of enamel were exposed to 12 ml of solution containing initially 1.3 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and constant 0.05 mM [F] and pH 7.0. The reactions of calcium, phosphate and fluoride are shown as a function of rate of solution flow through the reaction chamber.

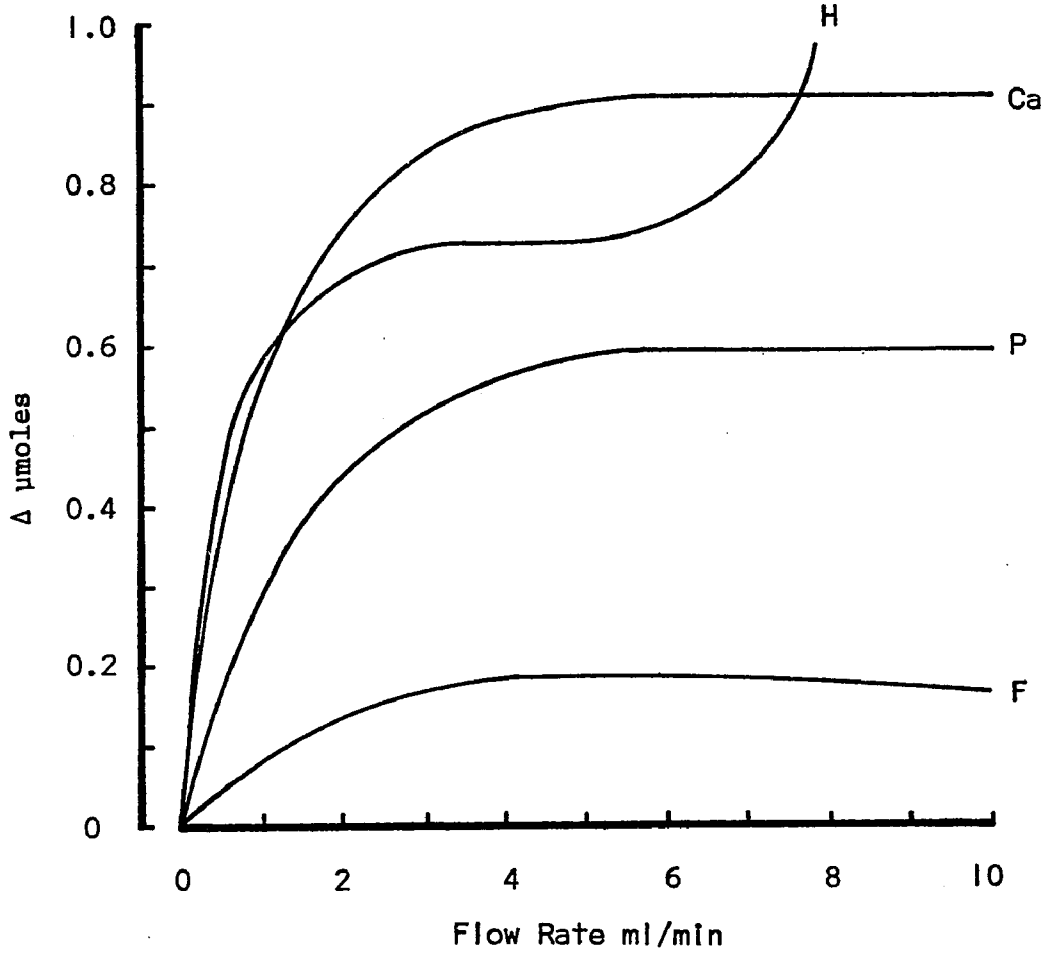

1.0
0.8
0.6
0.4
0.2
0
Δ µmoles
H
Ca
P
F
0
2
4
6
8
10
Flow Rate ml/min

TABLE 1.

Coefficients of Determination for Fit of the Assumed Models for the Variables lnCa, lnP, lnH, and lnF.†

Part A. 0 [F].

pH	lnCa	lnP	lnH
4.60	0.5209 (a)	0.9548	0.9888
	0.0041 (b)	0.0031	0.0071
5.00	0.2796	0.0579	0.9568
	0.0011*	0.0003	0.0011*
5.40	0.2723	0.0139	0.8983
	0.0283	0.0098	0.0012
5.80	0.0028	0.3149	0.1853
	0.0252	0.0023	0.0122
6.20	0.2075	0.4255	0.7769
	0.0288	0.0036	0.0036*
6.60	0.5483	0.2715	0.9256
	0.0118	0.0029	0.0034
7.00	0.6680	0.8430	0.9617
	0.0059	0.0012*	0.0098*
7.40	0.7820	0.6757	0.9359
	0.0148	0.0126*	0.0147*

† Coefficient of Determination = SS Regression/SS Total.

(a) Upper value in the table is the proportion of variation of the variable explained by the model.

(b) Lower value in the table is proportion given by lack of fit of the model.

Asterisks * indicate significance at the 0.05 probability level for lack of fit of assumed model.

TABLE 1. (continued)

Coefficients of Determination for Fit of the Assumed Models for the Variables lnCa, lnP, lnH, and lnF.†

Part B. With 0.05 mM [F].

pH	lnCa	lnP	lnH	lnF
4.60	0.6389 (a)	0.4452	0.9773	0.9563
	0.0047 (b)	0.0087	0.0003	0.0076*
5.00	0.1466	0.0145	0.9594	0.9663
	0.0060	0.0302	0.0001	0.0011
5.40	0.2597	0.5952	0.4821	0.9510
	0.0640*	0.0390	0.0023	0.0014
5.80	0.6270	0.9082	0.2537	0.9760
	0.0005	0.0044	0.2644*	0.0014
6.20	0.8847	0.8942	0.9511	0.9266
	0.0053	0.0017	0.0054*	0.0022
6.60	0.8188	0.8862	0.9645	0.9846
	0.0009	0.0051	0.0036	0.0018
7.00	0.9007	0.9647	0.9528	0.9469
	0.0291	0.0081*	0.0084*	0.0040*
7.40	0.8596	0.9166	0.9149	0.9527
	0.0106	0.0135	0.0100*	0.0180*

† Coefficient of Determination = SS Regression/SS Total.

(a) Upper value in the table is the proportion of variation of the variable explained by the model.

(b) Lower value in the table is proportion given by lack of fit of the model.

Asterisks * indicate significance at the 0.05 probability level for lack of fit of assumed model.

equations (3) and (5) yield good estimates of the variables lnCa, lnP, lnH and lnF.

Table 2 shows the mean values estimated from the least square curves of the variables $lnCa_o$, Ca rate, lnP_o, P rate, H_1, H rate, lnF_1, and F rate at pH 4.60, 5.00, 5.40, 5.80, 6.20, 6.60, 7.00 and 7.40 without fluoride or with 0.05 mM [F] (1 ppm). Table 3 shows the analyses of variance of the effect of fluoride concentration, pH and the interaction between fluoride and pH on the variables just mentioned.

Figure 7 shows the plots of the reaction rate of calcium as a function of pH in the presence of 0 or 0.05 mM fluoride in solution. Also shown in figure 7 are the upper and lower 95 percent confidence limits for both curves. Since the effect of the interaction between fluoride and pH on the calcium rates of reaction was significant ($p < 0.001$), the effect of fluoride is conditioned by the pH of the solution. Similarly, the effect of pH is conditioned by the concentration of fluoride. A fluoride concentration of 0.05 mM was more effective as accelerator of the calcium rate as pH was changed from 4.60 to 7.40. The calcium ion reaction rate decreased with higher pH levels from 0.068 at pH 4.60 to -0.210 at pH 7.40 in solutions with fluoride, and decreased from 0.047 at pH 4.60 to -0.369 at pH 7.40 in solutions containing 0.05 mM fluoride. The effect of 0.05 mM fluoride on the calcium phosphate reactions can be interpreted as the difference between the two calcium rate curves of figure 7 at the same rate value. In the acid side of the curve

TABLE 2.

Means and Standard Errors of the Variables $\ln Ca_o$, Ca Rate, $\ln P_o$, P Rate, H_1, H Rate, $\ln F_1$ and F Rate by pH and Fluoride Level.

pH	[F] ppm	$\ln Ca_o$	Ca Rate	$\ln P_o$	P Rate	H_1	H Rate	$\ln F_1$	F Rate
4.60	0	0.383 (a)	0.068	-0.202	0.101	-4.506	0.905		
		±0.003 (b)	±0.012	±0.007	±0.005	±0.117	±0.018		
	1	0.360	0.047	-0.208	0.026	-4.089	0.839	0.057	0.726
		±0.010	±0.004	±0.008	±0.013	±0.117	±0.012	±0.033	±0.018
5.00	0	0.401	0.052	-0.140	0.040	-2.512	0.879		
		±0.030	±0.003	±0.053	±0.005	±0.111	±0.014		
	1	0.394	0.014	-0.203	-0.007	-1.697	0.812	-0.049	0.711
		±0.014	±0.003	±0.021	±0.002	±0.083	±0.025	±0.031	±0.018
5.40	0	0.323	0.039	-0.224	0.005	-1.363	0.781		
		±0.040	±0.013	±0.023	±0.011	±0.100	±0.026		
	1	0.303	-0.023	-0.240	-0.056	-1.079	0.542	0.118	0.691
		±0.011	±0.003	±0.008	±0.007	±0.169	±0.033	±0.033	±0.018
5.80	0	0.376	0.003	-0.240	-0.027	-0.318	0.659		
		±0.019	±0.016	±0.021	±0.010	±0.088	±0.060		
	1	0.351	-0.073	-0.175	-0.092	0.368	1.256	0.042	0.634
		±0.022	±0.005	±0.015	±0.004	±0.070	±0.133	±0.030	±0.021

(a) Upper values in the table are means of 6 experiments.
(b) Lower values are standard errors of the mean.

TABLE 2. (continued)

Mean and Standard Errors of the Variables $lnCa_o$, Ca Rate, lnP_o, P Rate, H_1, H Rate, lnF_1 and F Rate by pH and Fluoride Level.

pH	[F] ppm	$lnCa_o$	Ca Rate	lnP_o	P Rate	H_1	H Rate	lnF_1	F Rate
6.20	0	0.331 ±0.015	-0.048 ±0.012	-0.230 ±0.026	-0.070 ±0.007	0.895 ±0.108	0.865 ±0.015		
	1	0.314 ±0.013	-0.116 ±0.006	-0.220 ±0.016	-0.145 ±0.004	0.353 ±0.082	0.963 ±0.032	-0.033 ±0.051	0.678 ±0.052
6.60	0	0.340 ±0.035	-0.104 ±0.021	-0.270 ±0.042	-0.06 ±0.014	2.492 ±0.130	0.680 ±0.028		
	1	0.258 ±0.047	-0.246 ±0.036	-0.289 ±0.037	-0.221 ±0.012	2.929 ±0.078	0.755 ±0.020	0.089 ±0.022	0.671 ±0.019
7.00	0	0.281 ±0.012	-0.181 ±0.028	-0.321 ±0.016	-0.180 ±0.007	4.066 ±0.118	0.635 ±0.014		
	1	0.288 ±0.027	-0.337 ±0.028	-0.258 ±0.014	-0.351 ±0.009	4.540 ±0.180	0.641 ±0.026	0.058 ±0.046	0.725 ±0.014
7.40	0	0.184 ±0.020	-0.210 ±0.013	-0.341 ±0.055	-0.208 ±0.010	5.782 ±0.282	0.705 ±0.025		
	1	0.269 ±0.016	-0.369 ±0.030	-0.295 ±0.029	-0.394 ±0.030	6.289 ±0.326	0.663 ±0.026	0.107 ±0.022	0.720 ±0.033

TABLE 3.

Analysis of Variance of the Effect of Fluoride and pH on the Variables $lnCa_o$, Ca Rate, lnP_o, P Rate, H_1, H Rate, lnF_1 and F Rate.

		Mean Squares			
		$lnCa_o$	Ca Rate	lnP_o	P Rate
[F]	1	0.0014 NS	0.1954***	0.0030 NS	0.2439***
pH	7	0.0354***	0.2145***	0.0291***	0.2040***
[F] X pH	7	0.0060 NS	0.0090***	0.0066 NS	0.0083***
Error	78	0.0035	0.0020	0.0047	0.0007

		Mean Squares			
		H_1	H Rate	lnF_1	F Rate
[F]	1	6.4183***	0.0424*		
pH	7	141.1077***	0.1975***	0.0225**	0.0063 NS
[F] X pH	7	0.0889 NS	0.1697***		
Error	78	0.1388	0.0106	0.0073	0.0043

NS - Not significant.

* - Significant at the 0.05 probability level.

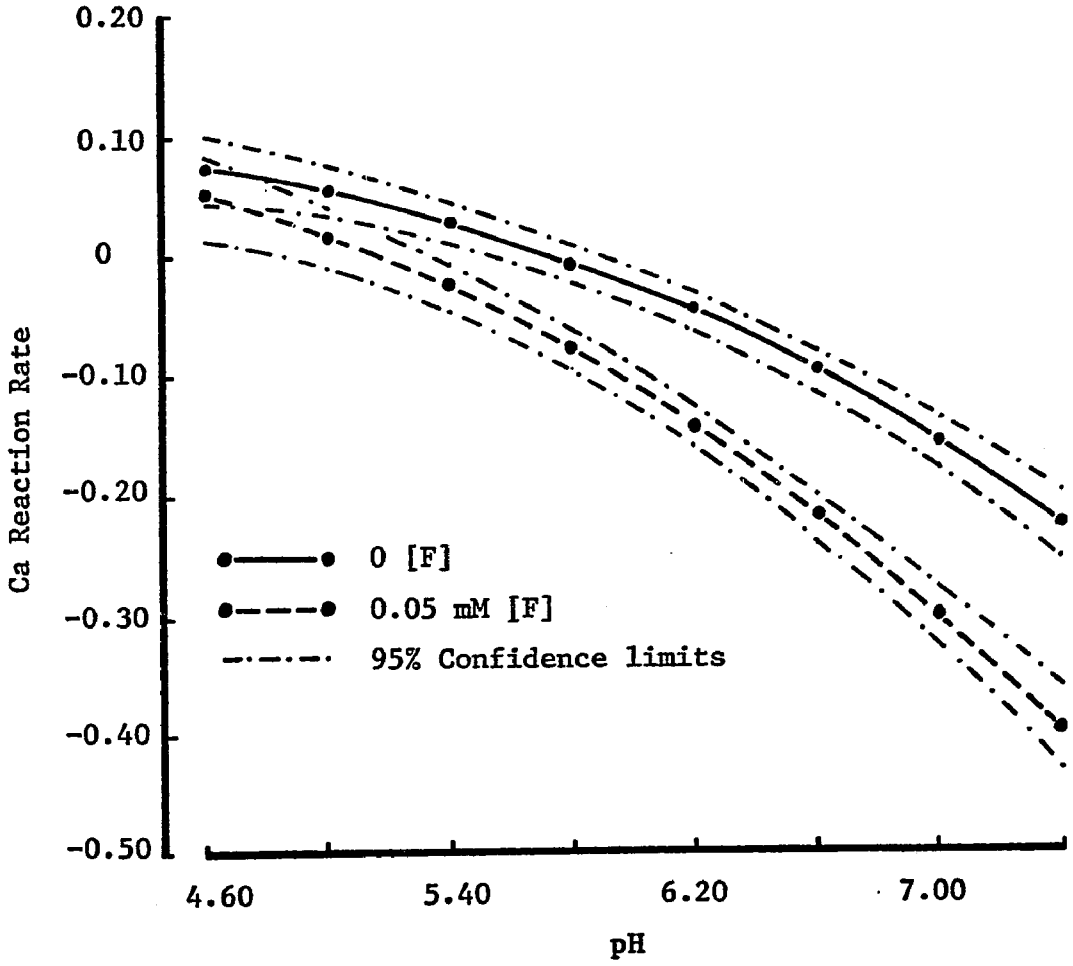
** - Significant at the 0.01 probability level.

*** - Significant at the 0.001 probability level.

Figure 7.

Reaction Rates of Calcium as a Function of pH with Upper and Lower 95 Percent Confidence Limits.

Twelve cm^2 of enamel were exposed to 12 ml of a solution initially containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0 or constant 0.05 mM [F] at constant pH 4.60, 5.00, 5.40, 5.80, 6.20, 6.60, 7.00 or 7.40. Each of the pH levels was replicated 6 times in experiments involving the use of fluoride and in experiments without fluoride. Duplicate samples for calcium analysis were drawn at half hour intervals.


0.20
0.10
0
-0.10
-0.20
-0.30
-0.40
-0.50
Ca Reaction Rate
0 [F]
0.05 mM [F]
95% Confidence limits
4.60
5.40
6.20
7.00
pH

(left) 0.05 mM fluoride had about the same effect on the calcium rates as an increase in pH of at least 0.4 units. At the higher pH levels, the effect of 0.05 mM fluoride was about the same as an increase in pH of 0.8 units.

Figure 8 shows the plots with 95 percent confidence limits of the phosphate reaction rate in solutions containing 0 or 0.05 mM fluoride as functions of pH. The effect of interaction between fluoride and pH on the rate of reaction of phosphate was significant ($p < 0.001$). A fluoride concentration of 0.05 mM was more accelerative on the phosphate rate as pH increased from 4.60 to 7.40. The phosphate reaction rate decreased with higher pH levels from 0.101 at pH 4.60 to -0.208 at pH 7.40 in solutions that did not contain fluoride, and from 0.026 at pH 4.60 to -0.394 at pH 7.40 in solutions containing 0.05 mM fluoride. The effect of fluoride on the phosphate rates was similar to the effect of increasing the pH by 0.7 units at the lower pH levels and by 0.9 at higher pH.

Figure 9 shows the relationship of the fluoride reaction rate with pH. The fluoride rate was 0.726 at pH 4.60 and 0.720 at pH 7.40 and, except for a decrease at pH 5.8, it remained relatively constant throughout the pH range studied. On analysis of variance the effect of pH on the fluoride rate of reaction was not significant.

Figure 8.

Reaction Rates of Phosphate as a Function of pH with Upper and Lower 95 Percent Confidence Limits.

Twelve cm^2 of enamel were exposed to 12 ml of solution initially containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl], and 0 or constant 0.05 mM [F] at constant pH 4.60, 5.00, 5.40, 5.80, 6.20, 6.60, 7.00 or 7.40. Each of the pH levels was replicated 6 times in experiments involving the use of fluoride and in experiments without fluoride. Duplicate samples for phosphate analysis were drawn at half hour intervals.

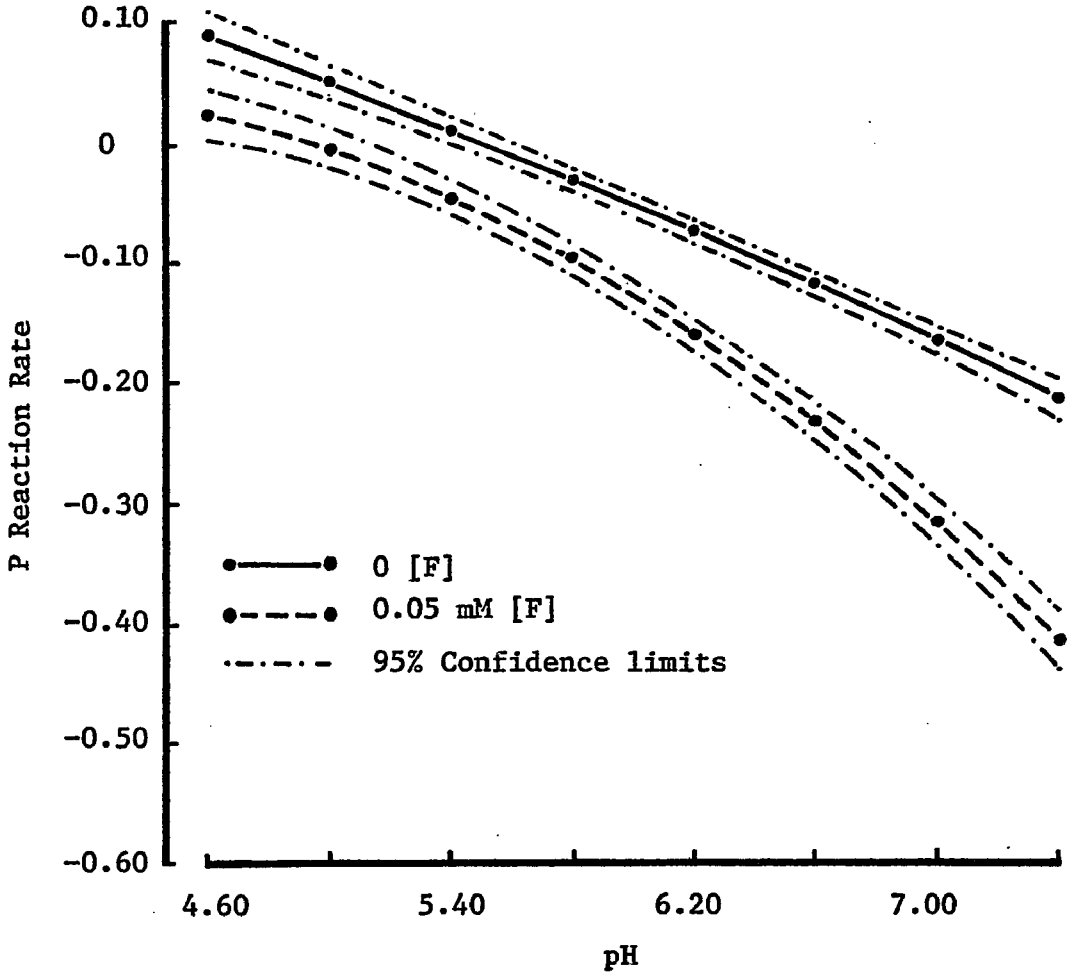
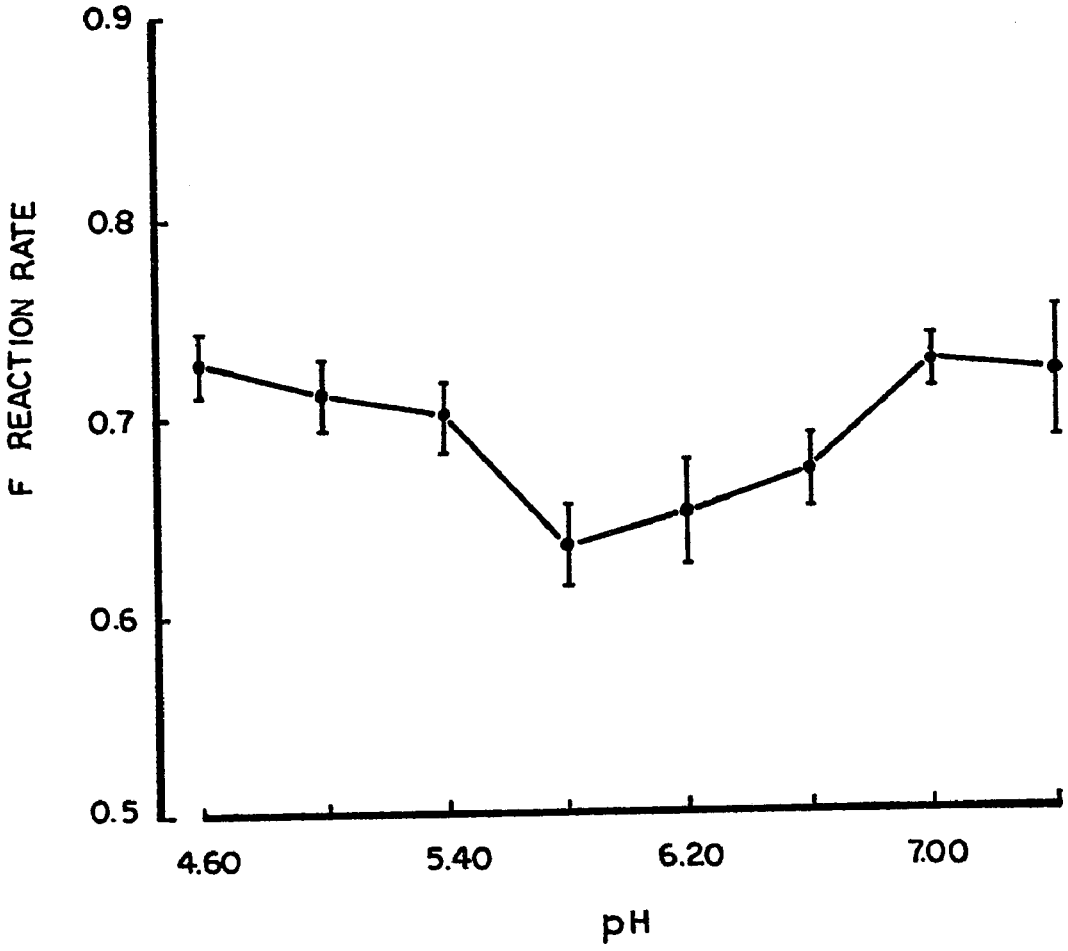

0.10
0
-0.10
-0.20
-0.30
-0.40
-0.50
-0.60
P Reaction Rate
0 [F]
0.05 mM [F]
95% Confidence limits
4.60
5.40
6.20
7.00
pH

Figure 9.

Reaction Rate of Fluoride as a Function of pH. Means and Standard Errors.

Twelve cm^2 of enamel were exposed to 12 ml of solution initially containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and constant 0.05 mM [F] at constant pH 4.60, 5.00, 5.40, 5.80, 6.20, 6.60, 7.00 or 7.40. Each pH level was replicated 6 times. Measurements of fluoride additions were made at half hour intervals.


0.9
0.8
0.7
0.6
0.5
F REACTION RATE
4.60
5.40
6.20
7.00
pH

Discussion

Reactions between the relatively large surface of enamel and the small volume of metastable solution induced mineral accretion in the interface. Release of hydrogen ions from the interface was associated with accretions of calcium phosphate mineral. The interfacial reactions of mineral accretion occurred in solutions containing less than 0.5 mM calcium, 0.3 mM phosphate, 0.05 mM fluoride, and pH 7.0. These concentrations are well below those that induce spontaneous precipitation in the bulk solution. However, accumulation of calcium and phosphate in the interface could possibly induce localized precipitations within and on the enamel surface. In order to differentiate growth of existing minerals from the precipitation and growth of new minerals in the enamel-solution interface, direct ultrastructural methods should be used. Surface accretions of minerals other than enamel minerals may prevent acid dissolution of the enamel tissue.

The reactions between the enamel and the solutions of neutral pH approached equilibrium after about 18 to 20 hours of interfacial reactions. The composition of the solutions containing 0.05 mM fluoride approximate the equilibrium solubility products of hydroxyapatites or fluorapatites. Therefore, the small fluoride ion concentrations apparently cause the accretion of calcium phosphates in the enamel-solution interface definable as calcium apatite. The rates of the interfacial reactions of calcium relative to the

reactions of phosphate and hydrogen ions in the solutions of neutral pH and 0.05 mM fluoride practically eliminate consideration of the formation of calcium phosphates other than hydroxyapatites or fluorapatites (McCann, 1968; Neuman and Neuman, 1958).

A fluoride ion concentration of 0.05 mM, i.e., 1 ppm, increased the rates of mineralization in the enamel-solution interface as compared to the rates in solutions without fluoride. These concentrations of fluoride caused: (1) increased concentration of calcium in the mineral accretions; (2) fluoride ion incorporation in the interfacial minerals; and (3) the accretions of minerals of lower solubility than the minerals formed in the interface in solutions without fluoride. The incorporation of fluoride ions in the interfacial minerals decreased the reaction ratios of H/Ca and H/P, and increased the reaction ratios of Ca/P, indicating the accretion of minerals that closely approximated the composition of the natural fluoride-containing calcium apatites. The fluoride ion effect was evident within 1 to 5 minutes of the initial time of enamel-solution interface, i.e., approximately the time required for equilibration of interfacial water with the experimental solution.

The effect of 0.05 mM fluoride ion concentrations on the interfacial reactions were further demonstrated in solutions with pH levels that ranged from 4.6 to 7.4, by increasing the rates of mineral accretions and decreasing the rates of demineralization. This level of fluoride in effect lowered the pH of apparent mineral accretion-demineralization equilibrium by about 0.4 pH units in mildly acid

solutions and by about 0.9 pH units in slightly basic solutions. The constant rates of fluoride ion incorporation in the interface over the pH range 4.6 to 7.4 may confirm initial adsorption of fluoride to the surface minerals (Volker, et al., 1940). The principal action of the adsorbed fluoride was to enhance the rate of formation of calcium accretions and to slightly decrease the amounts of base required to maintain the pH of the solutions. Such an action may contribute to the formation of fluorapatite or hydroxyapatite in the enamel-solution interface. However, the initially rapid catalytic action of the adsorbed fluoride may depend on displacement of water molecules or hydronium ions in the hydrated enamel-solution interface.

Flow of the solutions through the small chamber containing the enamel samples was necessary to transport reactants to and from the interface. Hydrogen ions were released in the interface as result of hydrolysis of $HPO_4^{=}$ and $H_2PO_4^{-}$ ions during calcium phosphate accretion (Neuman, Toribara and Mulryan, 1962). Measureable reactions of mineral accretion did not occur when the solutions were not recirculated through the interface. The rapid increase of the rates of mineral accretions with increased flow rates of solutions at the interface were related to the rates of hydrogen ion transport from the enamel-solution interface. Restriction of hydrogen ion diffusion during calcium phosphate mineral deposition causes the formation of gradients of more acid pH in the enamel surface than in the bulk solution. The rates of ionic diffusion within the interface

impose rate limitations on the reactions of the enamel-solution interface (Higuchi, et al., 1965).

The rates of mineral accretions sharply decreased when the hydrogen ions released from the interface were not titrated to constant pH in the mixing reservoir as the solutions were recirculated. The rates of base or acid titrations to maintain the pH of the recycling solutions provided a sensitive measure of the rates of phosphate reactions in the enamel-solution interface because the phosphate ions were practically the only hydrogen ion buffers in the system (Nancollas and Mohan, 1970). Increase or decrease of 0.1 pH unit in solutions of pH 7.0 caused about a tenfold change of the calcium phosphate activity product in solution (Neuman and Neuman, 1958). However, the rates of calcium phosphate mineralization increased only 1.5 times when the pH of the solutions were increased from pH 6.6 to pH 7.4. The disparity between the rates of mineral formation and the change of the calcium phosphate activity product in the solutions may show the effect of local accumulation of hydrogen ions in the enamel-solution interface.

The greater rates of calcium dissolution than phosphate dissolution in the solutions of pH 4.6 to pH 5.0 indicate the formation of a residual mineral surface richer in phosphate than in calcium content (Higuchi, et al., 1965). The reactions demonstrate hydrogen ion-calcium ion exchange in the enamel-solution interface as the mechanism of demineralization in these conditions of pH and calcium and phosphate concentration. These reactions may delineate

the mechanisms of incipient caries formation and the potential of small concentrations of fluoride ions to prevent the initiation of such demineralization. The 0.05 mM fluoride ion concentration in the solutions of this study prevented the surface demineralization and increased the rates of mineral formation equivalent to an average increase of about 0.7 pH units. This 0.7 pH unit may indicate the magnitude of the effect of fluoridated drinking water on reduction of dental caries. For example, if the critical pH of demineralization-remineralization equilibrium of the in situ mineral surface is pH 5.0, then a local concentration of 0.05 mM (1 ppm) fluoride may lower the critical pH of demineralization-mineralization equilibrium to pH 4.6 (Stephan, 1944).

Surfaces of enamel lightly acid-etched in weak acids have been described as softened (Head, 1912) or partially demineralized (Koulourides, 1968) enamel because the mild etching dissolves minerals within the surface rather than dissolving the entire mineral layering of the surface. These etched surfaces are soft and fragile. The softened surfaces reharden as calcium phosphate accretions occur in the interface between the residual minerals of the porous surface and the bulk phase of metastable non-precipitating solutions of calcium and phosphate (Feagin, et al., 1969; Koulourides, et al., 1961). Mineral accretions also occur within and on the etched enamel surface when immersed in solutions undergoing spontaneous precipitation of calcium phosphate. The precipitation or growth of minerals on the enamel surface may prevent acid

dissolution of the enamel tissue beyond the reaction times during which micropunch indention furnishes an adequate estimate of hardness of the enamel surface.

Summary

Kinetic studies of the reactions of the enamel-solution interface reveal possible mechanisms of action of factors which alter those reactions. Increase of the flow of solutions at the interface from 0.5 to 4 ml per minute markedly increased the rates of mineral accretion by removal of hydrogen ions released at the interface as consequence of calcium phosphate mineralization.

Fluoride ion concentrations of 0.05 mM caused: increased rates of mineral accretions; calcium enrichment of the minerals; fluoride ion incorporation in the minerals; and the formation of minerals of lower solubility than the minerals formed in the enamel-solution interface in solutions without fluoride. The fluoride ion effect was evident from the initial time of interface throughout the duration of interfacial reactions. The mechanism of the fluoride action was attributed to adsorption of fluoride in the interface in solutions of pH 4.6 to 7.4, which, in effect, neutralized hydrogen ions equivalent to at least 0.4 pH unit.

CHAPTER III

DENSITY AND X-RAY DIFFRACTION STUDIES OF ENAMEL SURFACE REACTIONS

Introduction

The natural formation of organic substances and minerals at the external surfaces of enamel increases the resistance of the tooth to dissolution in weak acid solutions (Miller, 1905). Early demonstrations that enamel surfaces, which had been softened in weak organic acids could be rehardened by saliva, suggested that remineralization was a reactive response to caries (Head, 1912). Later studies showed the potential of the fluoride ion for increasing the rate of enamel surface rehardening (Koulourides, Cueto, and Pigman, 1961).

The rehardening of acid-etched surfaces of enamel demonstrates increased resistance of the softened surfaces to the penetration of a diamond indenter (Hodge, 1936) which conditionally correlates with the deposition of calcium apatite within the surface (Feagin, Koulourides, and Pigman, 1969). The redeposition of apatite within enamel surfaces that had been partially demineralized by acid was also demonstrated by microradiography (Silverstone and Poole, 1969).

The method of microhardness testing provides a sensitive indirect measure of the mineralization of enamel surface. However, the accretion of potentially beneficial minerals on the surfaces of enamel causes apparent softening of the surfaces. The recently described technique of density gradient distribution of enamel mineral particles (Weidmann, Weatherell and Hamm, 1967) may provide a sensitive method for the separation and direct evaluation of the minerals deposited within and on surfaces of enamel.

The primary purpose of this study was to separate, by density gradient distribution, the aggregates of surface enamel and the products that resulted from reaction between the enamel and dilute acid solution before and after calcium phosphate mineral deposition on the acid etched surfaces. Our second purpose was to obtain X-ray diffraction identification of the density fractions separated from the enamel and its reaction products.

Materials and Methods

Preparation of the Enamel Samples.

Fifty-six permanent bovine incisor teeth that had no labial surface defects were selected and stored in distilled water at 4°C. Intact labial enamel surfaces, measuring 1 cm^2, were cut from the teeth under water spray in a thin sectioning machine with a diamond impregnated steel blade. The labial surfaces were smoothed with wet

pumice and a rubber dental prophylaxis cup and subsequently mounted on plastic blocks with dental sticky wax, leaving the labial enamel surfaces exposed. The pumiced surfaces were then acid-etched by immersion for 14 hours in slowly stirred 2 mM sodium acetate-acetic acid buffered to pH 5.0 at 37°C. After this, the acid-etched surfaces were immersed for 72 hours in metastable solutions of 1.5 mM [Ca], 0.9 mM [P], and 150 mM [NaCl] at 34°C. The solutions, which initially contained either 0.05 mM NaF or no NaF, were maintained at pH 7.0 by infusion of a solution of 50 mM NaOH automatically controlled by a pH potentiometer. The concentrations of calcium and phosphate in the metastable solutions were determined at the time of enamel surface immersion and at each 24 hour interval thereafter. In each experiment 28 surfaces of 1 cm^2 area each were immersed in a 500 ml volume of solution.

Powder was scraped from the labial surfaces of the enamel for density and X-ray diffraction studies. The pumiced, naturally mineralized surfaces were roughened with a diamond pencil and then scraped with a scalpel blade to obtain powder in the amount of about 60 mg weight from 56 of the 1 cm^2 surfaces. Powdered mineral was easily scraped with a scalpel blade from the acid-etched surfaces before and after 72 hour immersions in the metastable solutions. Approximately 60 mg of powder was obtained from 28 acid-etched surfaces. Powder samples of 200 mesh particles of human enamel were obtained by using the flotation-density method to separate enamel and dentin of ball-mill crushed teeth (Manly and Hodge, 1939). A

precipitate of calcium phosphate, obtained by mixing equal volumes of 13.5 mM $CaCl_2$ and 8.1 mM Na_2HPO_4 (pH 7.3, 22°C), was separated from the supernatant solution 1.5 hours after formation of the initial precipitate by millipore filtration. The precipitate was dried with a filtered stream of air at 22°C. Powder of a synthetic calcium apatite was obtained from a commercial source*. The powder from each of the various sample sources was dried to constant weight in room atmosphere. Selected samples were heated at 600°C for 24 hours.

Preparation of the Density Gradient Columns and Determination of Density.

Density gradient columns were prepared in 50 ml graduated cylinders by the modified method of Weidmann, et al. (1967). The height of the columns was about 12.5 cm (Fig. 10). Density gradient columns were prepared from mixtures of diiodomethane (DIM, sp. gr. 3.320 g/cm^3) and di-n-butyl phthalate (DBP, sp. gr. 1.052 g/cm^3).

For all samples except the precipitate a light (sp. gr. 2.50) and a heavy (sp. gr. 3.20) mixture was prepared with adequate proportions of DIM and DBP. For the determination of density of the precipitate, the density gradient column was prepared from mixtures having a density of 2.50 and 2.10.

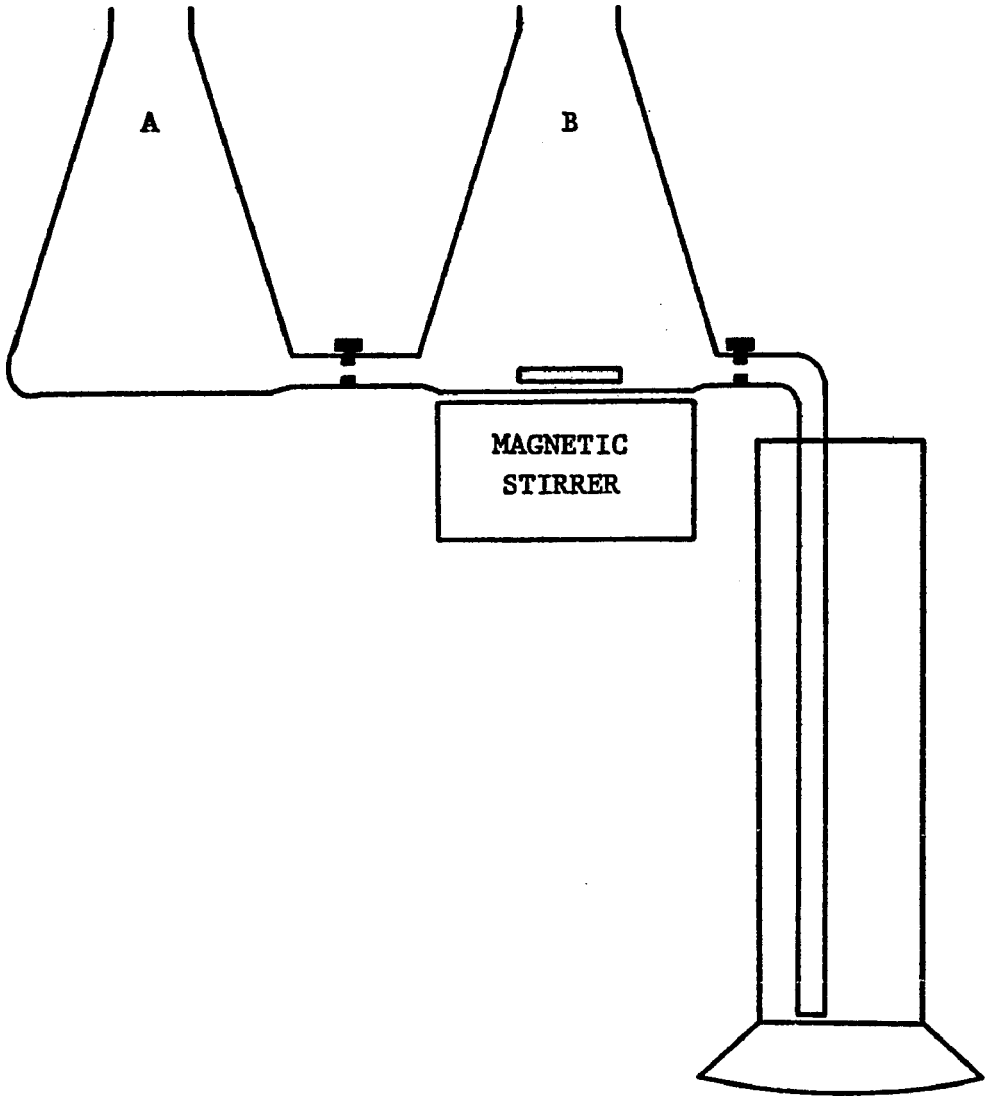
---

*Victor Chemical Company, Synthetic Apatite.

Figure 10.

Apparatus Used for the Preparation of Density Gradient Columns.

The apparatus consisted of a flask containing a dense liquid (A), a flask containing a light liquid (B), a magnetic stirrer, and a graduated column of uniform internal diameter.


A
B
MAGNETIC
STIRRER

Twenty-eight ml of the light fluid were placed in flask B and twenty-two ml of the heavy fluid were placed in flask A (Fig. 10). The connecting tap between the two flasks was opened momentarily to ensure that the pressures in the flasks were equal. The connecting tap was closed, the magnetic stirrer was activated and both taps were opened simultaneously so that the light fluid of flask B entered the gradient tube first. The light fraction was continuously raised by an influx of increasingly denser fluid at the bottom of the cylinder. The finished columns had almost linear density gradient between 2.50 to 3.20 or between 2.10 to 2.50. The density gradient columns were calibrated with sinkers of known density suspended permanently in the liquid at their density equilibrium points (Fig. 11).

Twenty-four mg of the mineral powder sample were dropped in the density column and allowed to equilibrate 24 hours at a constant temperature of 22°C. The range of distribution of the powder was directly measured on the graduated cylinder. The average density of a powder sample was visually estimated from the distribution of the greatest amount of material in the column.

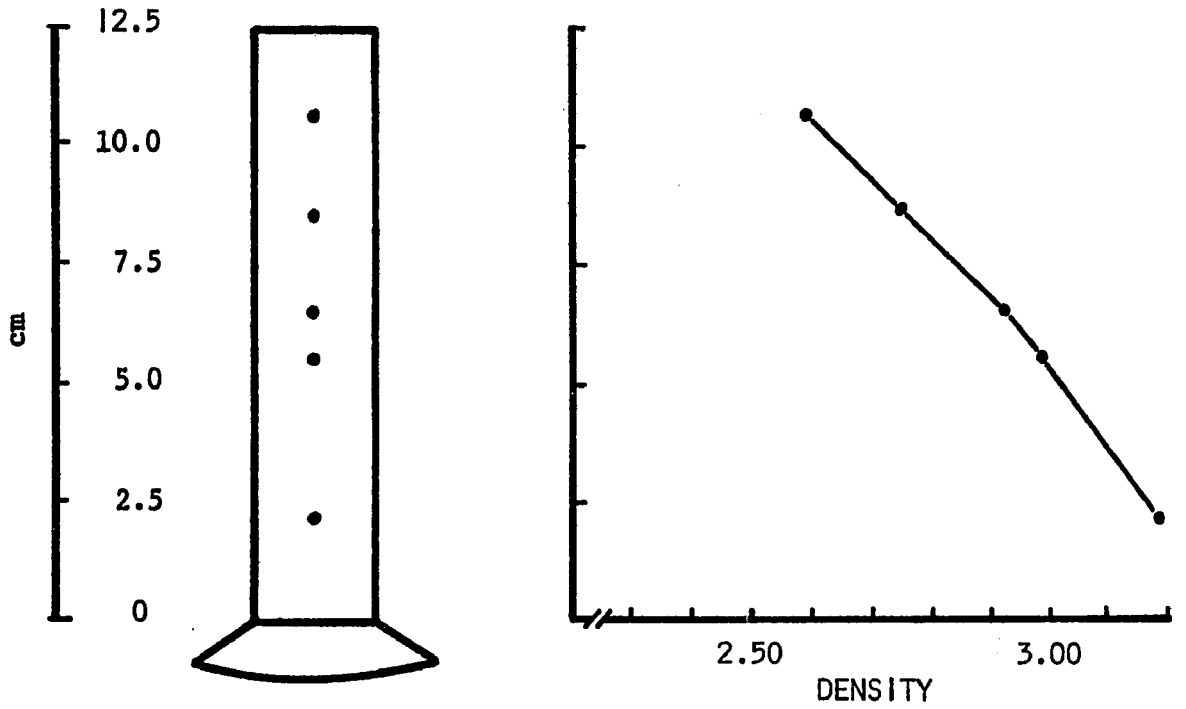
The powder sample was recovered with a thin pipette from the density gradient column on 0.45 micron pore sized paper filter*, then washed with acetone on the same filter.

---

*Whatman Filter Paper No. 42, Balston, Ltd., England. Average ash per circle of 18.5 cm in diameter: 0.00027 gr.

Figure 11.

Density Gradient Column Containing Sinkers of Known Density and Calibration Graph Showing Density Range and Slope.


12.5
10.0
7.5
5.0
2.5
0
cm
2.50
3.00
DENSITY

Procedure for X-ray Diffraction Analysis.

An aliquot of each powder sample was placed in a glass capillary tube of 0.3 mm inside diameter. This tube was slowly rotated in a Debye-Scherrer camera. The radius of the camera was 57.3 mm. The sample was exposed to nickel filtered copper K α radiation (1.54 Å wavelength) at 20 mAmps and 45 KV for 5 hours (Klug and Alexander, 1954). Reference diffraction patterns were obtained from untreated dental enamel and synthetic hydroxyapatite.

Results

Figure 12 shows the decreases of the calcium and phosphate concentrations in the metastable solutions during the 72 hour immersion of the acid-etched enamel surfaces. The concentrations of the calcium and phosphate were unchanged for several days when the surfaces were not immersed in the solutions. During the 72 hours that the surfaces (28 cm^2) of etched enamel were immersed in 500 ml of solution, calcium and phosphate concentrations slowly decreased 5% in solutions without fluoride and 10% in solutions with 0.05 mM NaF. The calcium and phosphate concentrations decreased within a range of 1.0 to 1.5% of each other on a rate basis.

Table 4 and figure 13 show the density distributions and estimated average densities of powder scraped from the surfaces of bovine enamel, 200 mesh particles of human enamel, a commercial apatitic mineral, and calcium phosphate minerals precipitated from

Figure 12.

Changes of Calcium and Phosphate Concentrations During Mineral Accretion at the Enamel-Solution Interface.

Twenty-eight cm^2 of enamel were immersed in 500 ml of solution containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0 or 0.05 mM [F]. The solutions were maintained at pH 7.0.

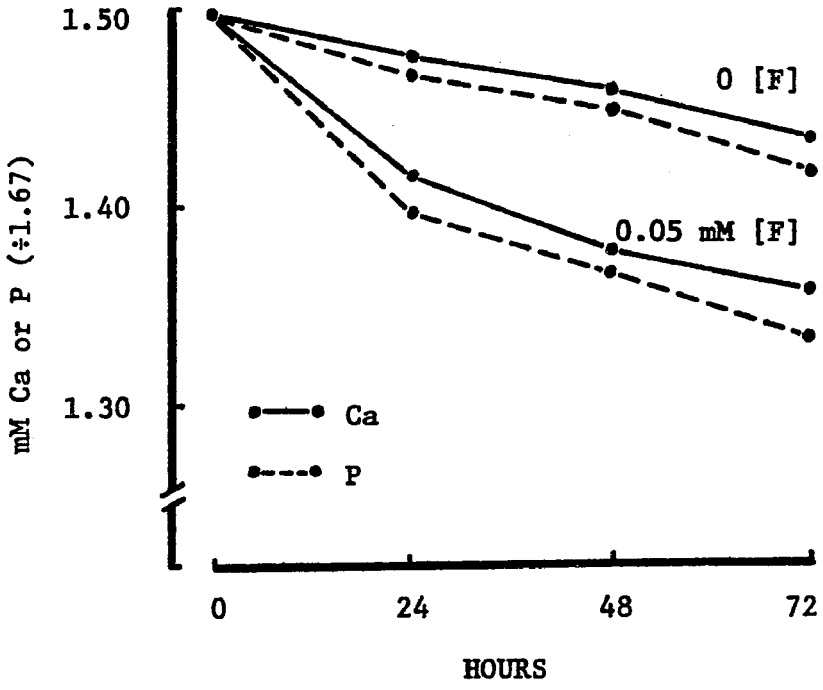

1.50
1.40
1.30
mM Ca or P (÷1.67)
0 [F]
0.05 mM [F]
Ca
P
0
24
48
72
HOURS

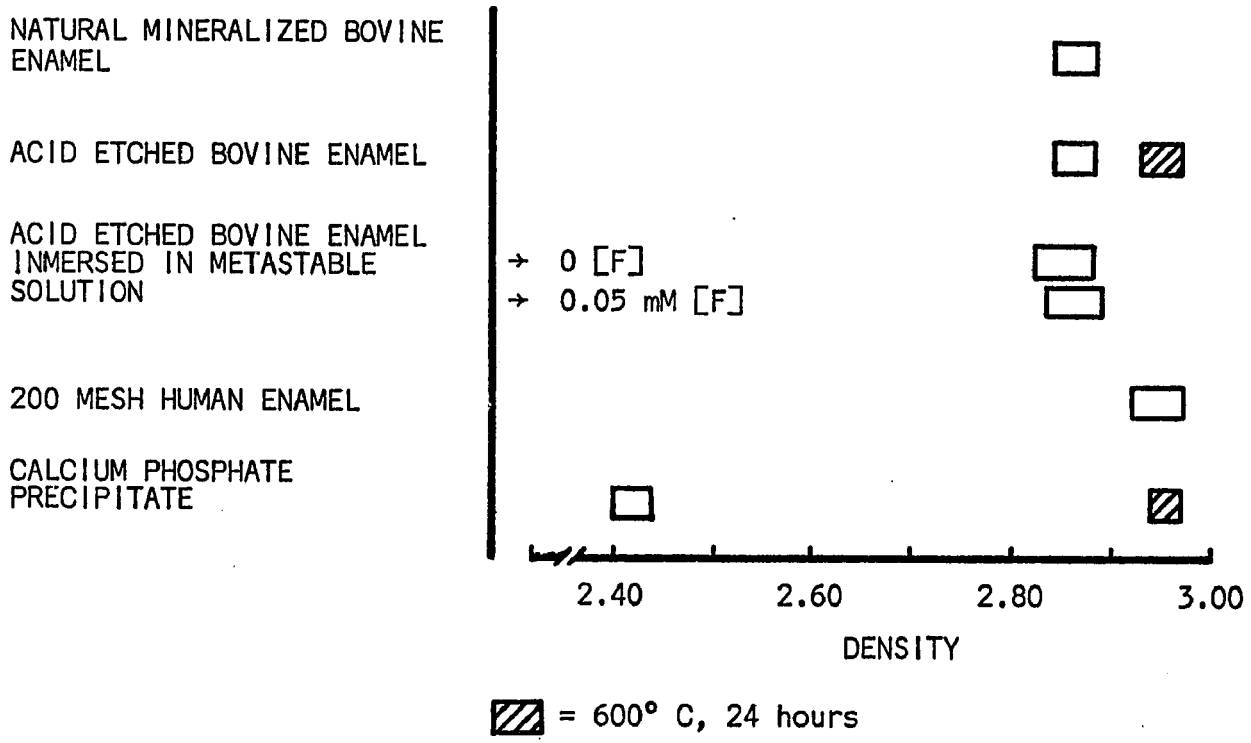
TABLE 4.

Density of Powder Scraped from Untreated and Experimentally Altered Enamel Surfaces, and Synthetic Apatite.

Sample	Density (g/ml)
Unaltered bovine enamel	2.836 - 2.885
Acid demineralized enamel	2.840 - 2.885
Acid demineralized enamel immersed in metastable solution without fluoride	2.815 - 2.880
Acid demineralized enamel immersed in metastable solution with 0.05 mM [F]	2.827 - 2.886
200-mesh human enamel	2.919 - 2.969
Apatitic precipitate	2.40 - 2.43

Figure 13.

Density of the Powder Scraped from Untreated and Experimentally Altered Enamel Surfaces, and Synthetic Apatites.


NATURAL MINERALIZED BOVINE ENAMEL
ACID ETCHED BOVINE ENAMEL
ACID ETCHED BOVINE ENAMEL INMERSED IN METASTABLE SOLUTION
→ 0 [F]
→ 0.05 mM [F]
200 MESH HUMAN ENAMEL
CALCIUM PHOSPHATE PRECIPITATE
2.40
2.60
2.80
3.00
DENSITY
= 600° C, 24 hours

solution. The powder scraped from the naturally mineralized bovine enamel and from the acid-etched bovine enamel equilibrated in a density range between 2.836 and 2.885. Powder scraped from the acid-etched surfaces, after a 72 hour immersion in the metastable solutions, equilibrated in density range of 2.815 to 2.880 and 2.827 to 2.886 both without and with the initial 0.05 mM NaF in solution, respectively. The estimated average densities of the bovine enamel powders were about 2.86. The density of the acid-etched bovine enamel increased from 2.86 to 2.94 after it was heated at 600°C for 24 hours. The density range of the 200 mesh particles of human enamel was between 2.919 and 2.969 without the heat treatment. After being heated at 600°C, the density of the commercial apatite increased from 2.63 to 2.83, and the density of the calcium phosphate precipitate increased from 2.41 to 2.95. A particle of natural mineral fluorapatite had a density of 3.10 (not shown). The densities of the powder samples were unchanged after a 24 hour suspension in the gradient columns.

Figure 14 shows the X-ray diffraction patterns of the powder scraped from the bovine enamel surfaces. The powder samples were recovered from the density gradient columns and washed with acetone before obtaining X-ray diffraction patterns of the various samples. There was not detected any diffraction line that did not correspond to the X-ray pattern of hydroxyapatite.

Figure 14.

X-ray Powder Diffraction Patterns of Enamel Surface Density Fractions.

A. Natural mineralized bovine enamel.
B. Acid-etched bovine enamel.
C. Acid-etched bovine enamel immersed in a solution containing 1.5 mM [Ca]; 0.9 mM [P]; 150 mM [NaCl] and 0 mM [F].
D. Acid-etched bovine enamel immersed in a solution containing 1.5 mM [Ca]; 0.9 mM [P]; 150 mM [NaCl] and 0.05 mM [F].
E. 200 mesh human enamel.
F. Calcium phosphate spontaneously precipitated from a solution containing 6.75 mM [Ca] and 4.05 mM [P] at pH 7.3.

Discussion

Apatites closely similar to hydroxyapatite were deposited as crystal growth on enamel surfaces during immersion in metastable solutions (Feagin, Gonzalez and Jeansonne, 1972). The decreases of the calcium and phosphate concentrations in the metastable solutions indicate the rates of mineral accretion on the enamel surfaces. The rate of mineral accretion was slow because of the large volume of solution in relation to the number of immersed enamel surfaces. The effect of the fluoride ion obviously increased the rate of mineral accretion even though the concentration of this ion in the solution was depleted during the first 24 hours of the reactions (Thomas and Feagin, 1973).

It was nearly impossible to scrape powder from the pumiced surfaces of naturally mineralized bovine enamel with a steel scalpel blade, but scraping powder was facilitated after light scoring with a diamond pen. In contrast, the acid-etched minerals were easily separated from the underlying sound minerals with a sharp scalpel blade because the sound enamel was harder than the stainless steel blade (Hodge, 1936). The minerals deposited on the surface were also relatively soft. Assuming that the rates of calcium deposition indicate the rates of apatite formation, approximately 6 mg apatite were deposited on the enamel surfaces that were immersed in the solutions containing fluoride, and about 3 mg apatite were deposited on the surfaces immersed in the solutions that contained no fluoride.

Therefore, the minerals deposited on the surfaces account for about 10% of the 60 mg powder scraped from the 12 cm^2 of enamel surfaces.

The average density of the minerals scraped from the bovine enamel surfaces of 2.86 approximates the density of 2.88 estimated for normal hydrated human enamel (Angmar, Carlström and Glas, 1963). The 600° heat treatment increased the density of the bovine minerals to approximately the same density range as that of the 200 mesh particles of human enamel. The higher density of the 200 mesh human enamel probably resulted from dehydration and crystal strain that occurred during ball-mill preparation (Weber and Posner, 1967). The minerals were surgically scraped from the bovine enamel to avoid the heat and strain that might be imposed on the mineral crystals by rotary instruments.

The almost identical density ranges of the powder scraped from the enamel surfaces both before and after acid-etching indicates that composition of the enamel minerals and the acid-etched enamel minerals were essentially alike. Therefore, the minerals of the etched surfaces were largely stoichiometric in relation to enamel, as previously indicated by determination of the ratios of the calcium and phosphate dissolved from the surfaces (Feagin, et al., 1969).

The amount of the powder scraped from the surfaces after their immersion in the metastable solutions that equilibrated at the lower end of the respective density ranges probably represents the small amounts of minerals deposited in the acid-etched enamel. This

mineral, which has a slightly lower density than the etched enamel minerals may occur as smaller crystals with greater hydration than the enamel minerals (Carlström, Glas and Angmar, 1963). The fluoride ions probably increased the crystallite size of only a small percentage of the minerals deposited on the surfaces (Schraer, et al., 1962), because the fluoride was rapidly removed from the solution during the early stage of the reactions. Perhaps the effect of fluoride on the mineral crystallinity as measured by density is small because the minerals deposited on the surfaces account for about 10% of the powder scraped from the surfaces.

The calcium phosphate minerals recently precipitated from the supersaturated, unstable, solution were included in this study to show that the poorly crystallized calcium phosphates (Watson and Robinson, 1953) were of stable density during suspension in the density gradient fluids. The density of the precipitated calcium phosphates was stable between 2.40 and 2.44 during the 24 hour period of suspension in the density fluids. The increased density of the precipitates after the 24 hour heat treatment largely represents the loss of water from the minerals. The density ranges of the heated samples of the enamel minerals, which were slightly lower than ranges of the heated precipitate, may reflect the presence of trace minerals or other contaminants in the enamel that were not present in the precipitates.

The X-ray diffraction patterns of the various enamel samples and the calcium phosphate precipitates were similar to

hydroxyapatite patterns. Since all patterns observed showed only diffraction lines that corresponded to a hydroxyapatite pattern, it can be stated that the only crystalline phase present in significant amounts in all samples was apatite. However, the amorphous minerals that were probably present in the low density precipitates may not be apparent by the powder X-ray diffraction methods used in this work (Klug and Alexander, 1954). The degree of crystallinity of the samples was not determined quantitatively although studies of this kind should be desirable.

Summary

The density gradient distribution of powder scraped from the outer labial surfaces of pumiced bovine incisor teeth was 2.84 to 2.88 g/cm^3, both before and after mild etching of the surfaces in weak acid. The densities of powder were between 2.81 and 2.88, when scraped from the acid-etched surfaces after a 72 hour immersion in metastable solutions that contained no fluoride. The powder was in the density range of 2.83 to 2.90 when scraped from surfaces that had been immersed in the solutions that initially contained 0.05 mM NaF. The close range in the densities demonstrates almost identical compositions of the powder samples scraped from the naturally mineralized and the experimentally altered enamel surfaces, except for small amounts of minerals that were deposited on the surfaces which had been immersed in metastable solutions that contained no fluoride.

The X-ray diffraction patterns of the enamel powder and the low density calcium phosphate precipitates were all apatitic.

CHAPTER IV

ELECTRON MICROSCOPY OF THE ENAMEL SURFACE BEFORE AND AFTER MINERALIZATION REACTIONS

Introduction

Enamel caries begins as demineralization of the enamel mineral surface in weak acid solutions. The initial demineralization is probably not recognized because of the dynamic interactions of the erupted mineral surface with the oral fluids (Speirs, 1971). However, post-eruptive alterations of the more superficial enamel tissue indicate previous demineralizations and mineral depositions (Little and Steadman, 1966). The in vitro weak acid demineralized, softened, surfaces of enamel regain hardness in the more superficial 10 microns as calcium phosphate minerals deposit in the etched surfaces (Feagin, Koulourides and Pigman, 1969). Similar calcium phosphate mineral accretions occur in scratches on polished surfaces of enamel. This shows induction of apatite crystal growth by fully mineralized enamel tissue simply by increase of the surface area rather than mineralization of hypomineralized enamel. The in vitro studies show the potential reactivity of the in situ enamel mineral surface (Feagin, Gonzalez and Jeansonne, 1972; Gonzalez, Jeansonne and Feagin, 1973).

The transmission (TEM) and scanning (SEM) electron microscopes have been used to study the ultrastructure of enamel tissue before and after reactions in acid solutions (Frank, 1967; Johnson, Poole and Tyler, 1971), and after immersion in metastable solutions of calcium phosphate and carbonate (Muhlemann, Lenz and Rossinsky, 1964). The SEM showed the formation of a mineral deposit on acid-demineralized surfaces of enamel after lengthy immersion in unstable solutions of calcium phosphate (Zuniga and Koulourides, 1971). The TEM suggested the deposition of calcium carbonate phosphate minerals in intercrystalline areas as much as 100 microns deep in etched surfaces of enamel (Muhlemann, et al., 1964). Selective deposition of calcium phosphate fluoride minerals within or on tooth surfaces may inhibit the enamel caries process.

The purpose of this study was to determine the SEM and TEM appearance of lightly acid-etched surfaces of enamel before and after calcium phosphate mineral deposition at the surfaces. Lightly abraded labial enamel surfaces of bovine incisor teeth were etched in weak acid and then immersed in solutions containing calcium and phosphate concentrations capable of supporting apatite mineral growth as described in previous chapters.

Materials and Methods

Preparation of the Enamel Surfaces.

Enamel slabs with 0.25 cm^2 labial surface were cut from bovine

incisor teeth in a thin sectioning machine. Each slab was mounted on a plastic block with dental sticky wax leaving only the labial enamel surface uncovered. The surfaces were lightly abraded with wet silicon carbide (#600 grit) and wet pumice with a dental prophylaxis cup. The pumiced surfaces were acid-etched in 2 mM sodium acetate-acetic acid solution at pH 5.0 and 37°C with slow stirring. In each experiment twelve surfaces (3 cm^2) were immersed in 500 ml of the acetate buffer for 14 hours and then rinsed in deionized water. Nine (2.25 cm^2) of the etched surfaces were immersed in 500 ml of a solution containing 1.5 mM [Ca], 0.9 mM [P], 0.05 mM [F] and 150 mM [NaCl] at 34°C. The pH of the solution was constantly maintained at pH 7.0 with an automatic titrator (pH stat) and the solution was continually mixed with a rate magnetic stirring of about 160 r.p.m. Randomly selected enamel slabs, and micro-samples of solution for calcium (Sarkar and Chauhan, 1967) and phosphate (Chen, Toribara and Warner, 1956) analysis, were withdrawn at the 2, 4, and 8 hour times of reaction. In other experiments acid-etched surfaces were immersed in the solution for several days. In one study etched surfaces were immersed in unstable solution containing 6.75 mM [Ca] and 4.05 mM [P]; pH of the solution was 7.3. Temperature was 25°C.

Preparation of the Enamel Surfaces for Transmission Electron Microscopy.

The enamel surfaces for TEM study were dehydrated 5 minutes

in absolute ethanol immediately after removal from the experimental solutions in order to stop further contact between the surfaces and the solution and avoid drying of the enamel surfaces. After dehydration the enamel samples were transferred successively to a 50 percent solution of propylene oxide and ethanol and to 100 percent propylene oxide in steps of 5 minutes each. Infiltration of the samples was made with a solution of equal volumes of propylene oxide and araldite*, the embedding resin (Luft, 1961) for 16 hours.

Embedding was made with araldite under partial vacuum at polymerization temperatures that varied from 25°C to 60°C. Sections of the embedded enamel slabs of about 0.3 mm thick were cut perpendicular to the labial surface in an incisal-cervical direction with a thin sectioning machine. These pieces of enamel were embedded in araldite, polymerized as before (Fig. 15A). Part of the block containing the enamel sample was removed with a carborundum disk in the manner indicated in the shaded portion of figure 15B. The side of the enamel face that touched the diamond knife first was left uncovered by araldite (Fig. 15C). Enamel was cut in an ultramicrotome** in 900 Å sections again perpendicular to the labial surface with a diamond knife of included angle of 49° set a 3° clearance angle (Fernandez-Moran, 1953). Estimated sectioning velocity was 4 mm/sec. The thin sections were removed from the water filled

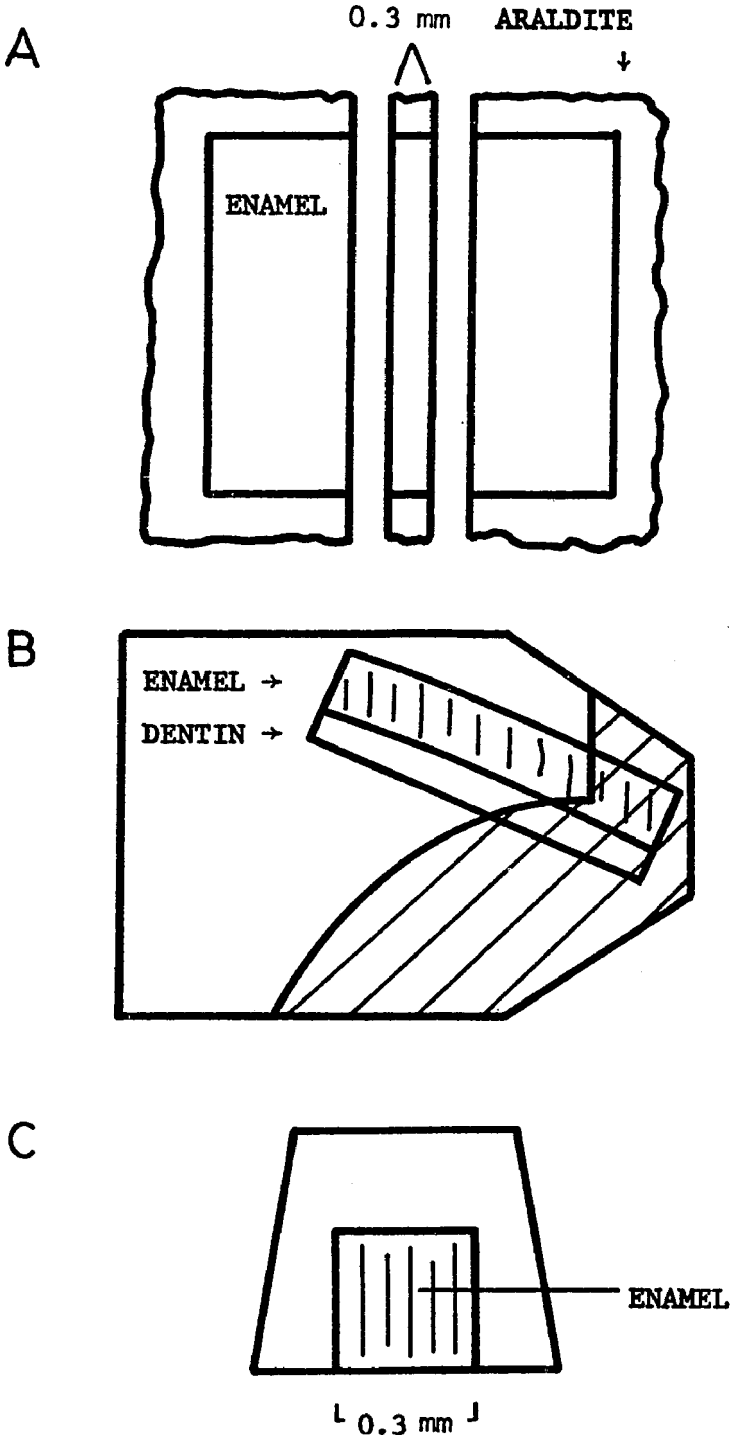
---

*Araldite 502, Ciba Products, N. Y.
**Sorvall Porter-Blum MT-2 ultramicrotome.

Figure 15.

Diagram of the Preparation of Enamel for Ultramicrotomy.

A. The whole enamel surfaces were dehydrated in ethanol, infiltrated in a mixture of araldite and propylene oxide and embedded in araldite. Thick sections of 0.3 mm width of the embedded block were obtained in a thin sectioning machine equipped with a diamond impregnated steel blade.
B. The thick sections of enamel were again embedded in araldite and abraded with a carborundum disk in the manner indicated (shaded).
C. In the surface of the block to be cut with the diamond knife, the enamel was surrounded by araldite in all sides, except in the border that first touched the knife.


A
0.3 mm
ARALDITE
ENAMEL
B
ENAMEL →
DENTIN →
C
ENAMEL
0.3 mm

collection trough as quickly as possible on 200 mesh, carbon-parlodion coated, copper grids for examination in the electron microscope at 80 KV. The thin sections were studied and photographed at several magnifications, however, for comparison TEM photographs of 14,000 and 82,000 magnifications are shown in this study. Electron diffraction patterns were obtained within 3 μ from the labial surfaces of the enamel.

Preparation of the Enamel Surfaces for Scanning Electron Microscopy

For examination in the scanning electron microscope, the enamel surfaces were dehydrated in absolute ethanol and coated with an alloy of 60 percent gold and 40 percent palladium (Au-Pd coat) in a high vacuum evaporator*. The calculated thickness of the Au-Pd layer was about 500 Å. The enamel surfaces were studied and photographed in a scanning electron microscope** operated at 20 KV.

Results

Table 5 shows the decreases of calcium and phosphate in the metastable solution during mineral reactions on the etched surfaces of enamel. Apparently 8.9 μmoles Ca and 4.4 μmoles P deposited per cm^2 enamel surface in the first 2 hours, and 22.2 μmoles Ca and

---

* Hitachi.
**Stereoscan Mark II, Cambridge.

TABLE 5.

Reactions of Calcium and Phosphate at Surfaces of Acid* Etched Enamel Immersed in Metastable Solution of Constant pH 7.0

	μmoles/500 ml in Solution			μmoles/cm^2 Deposited	
Hour	Calcium	Phosphate	cm^2 Surface Immersed	Calcium	Phosphate
0	750 (1.50 mM)	450 (0.90 mM)	2.25	--	--
2	730 (1.46 mM)	440 (0.88 mM)	2.25	8.9	4.4
4	710 (1.40 mM)	426 (0.85 mM)	1.50	22.2	13.6

*14 hour immersion in 2 mM sodium acetate-acetic acid solution at pH 5.0 dissolved 31 μmoles/cm^2 Ca and 24 μmoles/cm^2 P from pumiced enamel surfaces.

13.6 μmoles P deposited per cm^2 in 4 hours immersion of the etched surfaces in the solution. The decreases of Ca and P were too small for accurate determination when only 0.72 cm^2 surfaces were immersed in the 500 ml solution. In other similar experiments about 24 μmoles/cm^2 Ca and 14 μmoles/cm^2 P deposited on enamel surfaces immersed in the solution 96 hours. An average of 31 μmoles/cm^2 Ca and 24 μmoles/cm^2 P dissolved from the pumiced surfaces during the 14 hour immersion in the acetate buffer.

Figure 16 shows the TEM appearance of the outermost 10 microns of the lightly abraded labial enamel surface of bovine incisors. The general ultrastructural features of enamel tissue are apparent regardless of the numerous fractures in the section. The embedding material did not penetrate the labial surface of the fully mineralized tissue which caused difficulty in obtaining unshattered sections.

Figure 17 shows the TEM appearance of the labial surface of the enamel after 14 hour immersion in the acetate-buffered weak acid solution. Partial and complete dissolution of the enamel mineral crystals occurred in the outer 5 to 9 microns of the surface. Figure 18 shows relatively large areas of complete structural dissolution in the central portions of the prisms beginning about 3 microns beneath the surface and extending to about 7 microns deep in some areas of the etched surfaces. Some of the areas of complete mineral dissolution were open to the surface. The subsurface holes were filled with the embedding material. The prismatic structure of enamel was still observed after 14 hours of demineralization.

Figure 16.

Transmission Electron Micrograph of Lightly Abraded Labial Enamel Surface of Bovine Incisors.

The plane of sectioning was perpendicular to the surface. The bar indicates the length of 1 μ.

Figure 17.

Transmission Electron Micrograph of the Labial Surface of Enamel After 14 Hour Immersion in a Solution of 2 mM Sodium Acetate Buffer at pH 5.0.

The plane of sectioning was perpendicular to the surface. The bar indicates the length of 1 μ.


16


17

Figure 18.

Transmission Electron Micrograph of the Labial Surface of Enamel After 14 Hour Immersion in a Solution of 2 mM Sodium Acetate Buffer at pH 5.0.

The plane of sectioning was perpendicular to the surface. The bar indicates the length of 1 μ.

Figure 19.

Transmission Electron Micrograph of the Labial Surface of Acid-Etched Enamel After 4 Hour Reaction with a Metastable Calcifying Solution.

Acid-etched surfaces of enamel were immersed 4 hours in a solution of 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0.05 mM [F]. Solution pH was maintained at 7.0. The bar indicates the length of 1 μ.


18


19

Figure 19 shows the TEM appearance of a section of acid-etched surface after 4 hour immersion in the metastable solution. The samples of this group did not exhibit areas of holes in contrast to the etched surfaces (Fig. 18). Figure 20 shows the TEM appearance of the acid-etched surfaces after 8 hour immersion in the metastable solution, which is practically indistinguishable from surfaces after 4 hour immersions.

Figure 21 shows the TEM appearance of etched enamel surfaces immersed 4 days in the calcium-phosphate solution. The outermost 6 microns of the surface is unlike the acid-etched surface and etched surfaces exposed for short times to calcium phosphate solutions. The transition between the deeper normal crystals and the acid-etched crystals is replaced by a sharp line between normal enamel mineral and a new material. The deposited material appeared unlike the enamel tissue ultrastructure.

Figure 22 shows the TEM appearance of the mineral deposited on surfaces of enamel during spontaneous precipitation within the bulk solution. The precipitate appeared fibrous and disorganized. It was not similar in appearance to enamel minerals or the minerals deposited in the etched surfaces from metastable solutions. The crystal size of this mineral was small compared to the normal enamel crystals.

Figure 23 shows the TEM appearance at 82,000 magnifications of normal crystals of enamel. The crystals that were cut longitudinally appear rectangular and have uniform electronlucency.

Figure 20.

Transmission Electron Micrograph of the Labial Surface of Acid-Etched Enamel After 8 Hour Reaction with a Metastable Calcifying Solution.

Acid-etched surfaces of enamel were immersed 8 hours in a solution of 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0.05 mM [F]. Solution pH was maintained at 7.0. The bar indicates the length of 1 μ.

Figure 21.

Transmission Electron Micrograph of the Labial Surface of Acid-Etched Enamel After 4 Day Reaction with a Metastable Calcifying Solution.

Acid-etched surfaces of enamel were immersed 4 days in a solution of 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0.05 mM [F]. Solution pH was maintained at 7.0. The bar indicates the length of 1 μ.


20

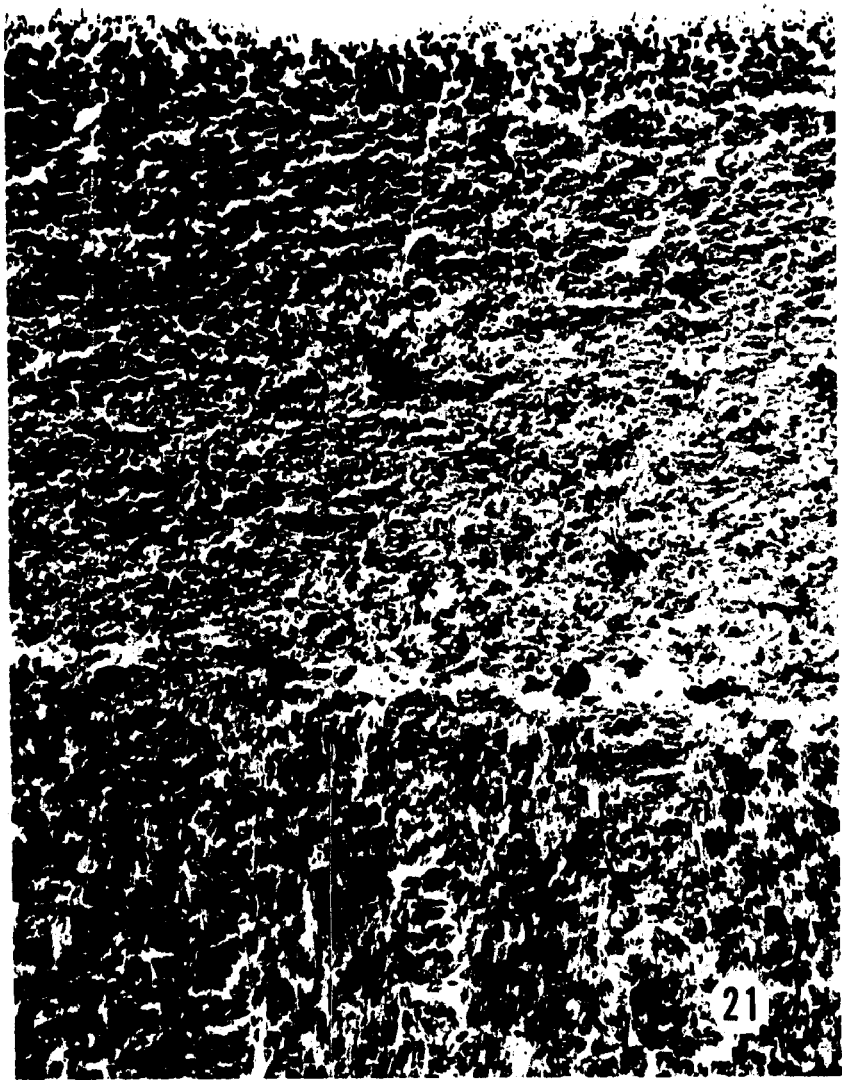

21

Figure 22.

Transmission Electron Micrograph of the Mineral Deposited on the Surfaces of Enamel After Spontaneous Precipitation of a Calcium Phosphate Solution.

Acid-etched surfaces of enamel were immersed in a solution containing 6.75 mM [Ca] and 4.05 mM [P]. Initial pH was 7.3. The bar indicates the length of 1 μ.


22

Figure 24 shows the TEM appearance at 82,000 magnifications of acid-etched crystals of enamel. They appear smaller, less angular and less electron dense than deeper unreacted crystals.

Figure 25 shows the TEM appearance at 82,000 magnifications of the mineral deposited on etched surfaces of enamel after 96 hours immersion in a metastable solution of calcium phosphate. The crystals have a smaller crystallite size than normal crystals and present irregular crystallite borders. The electronlucency of these crystals was not uniform.

Figure 26 shows the TEM appearance at 82,000 magnifications of the mineral deposited on the surfaces of enamel after spontaneous precipitation from an unstable calcium phosphate solution. The precipitate appears disorganized. Its crystals have needle-like shape. The crystal size was clearly smaller than the crystal size of any other mineral observed herein.

Figure 27 shows electron diffraction patterns of selected areas 1 micron in diameter about 3 microns below the labial enamel surfaces. Figure 27A shows the electron diffraction patterns of sections of enamel after complete demineralization on the TEM grid, i.e., the electron diffraction pattern of the residual organic material. Figures 27B and 27C show practically identical electron diffraction patterns for the selected areas of normal and acid-etched enamel. Figure 27D shows electron diffraction patterns of low granularity, and evenly spaced diffractions around the patterns, for the etched surfaces after 4 day immersion in the metastable

Figure 23.

Transmission Electron Micrograph of Untreated Bovine Enamel.

The bar indicates the length of 1000 Å.

Figure 24.

Transmission Electron Micrograph of Dental Enamel Reacted with a Solution of 2 mM Sodium Acetate Buffer at pH 5.0.

The bar indicates the length of 1000 Å.

Figure 25.

Transmission Electron Micrograph of the Mineral Formed on the Enamel Surface After 4 Days of Reaction with a Metastable Calcifying Solution.

Acid-etched surfaces of enamel were immersed 4 days in a solution initially containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0.05 mM [F]. Solution pH was maintained at 7.0. The bar indicates the length of 1000 Å.

Figure 26.

Transmission Electron Micrograph of the Mineral Deposited on the Surfaces of Enamel After Spontaneous Precipitation of a Calcium Phosphate Solution.

Acid-etched surfaces of enamel were immersed in a solution containing 6.75 mM [Ca] and 4.05 mM [P]. Initial pH was 7.3. The bar represents the length of 1000 Å.

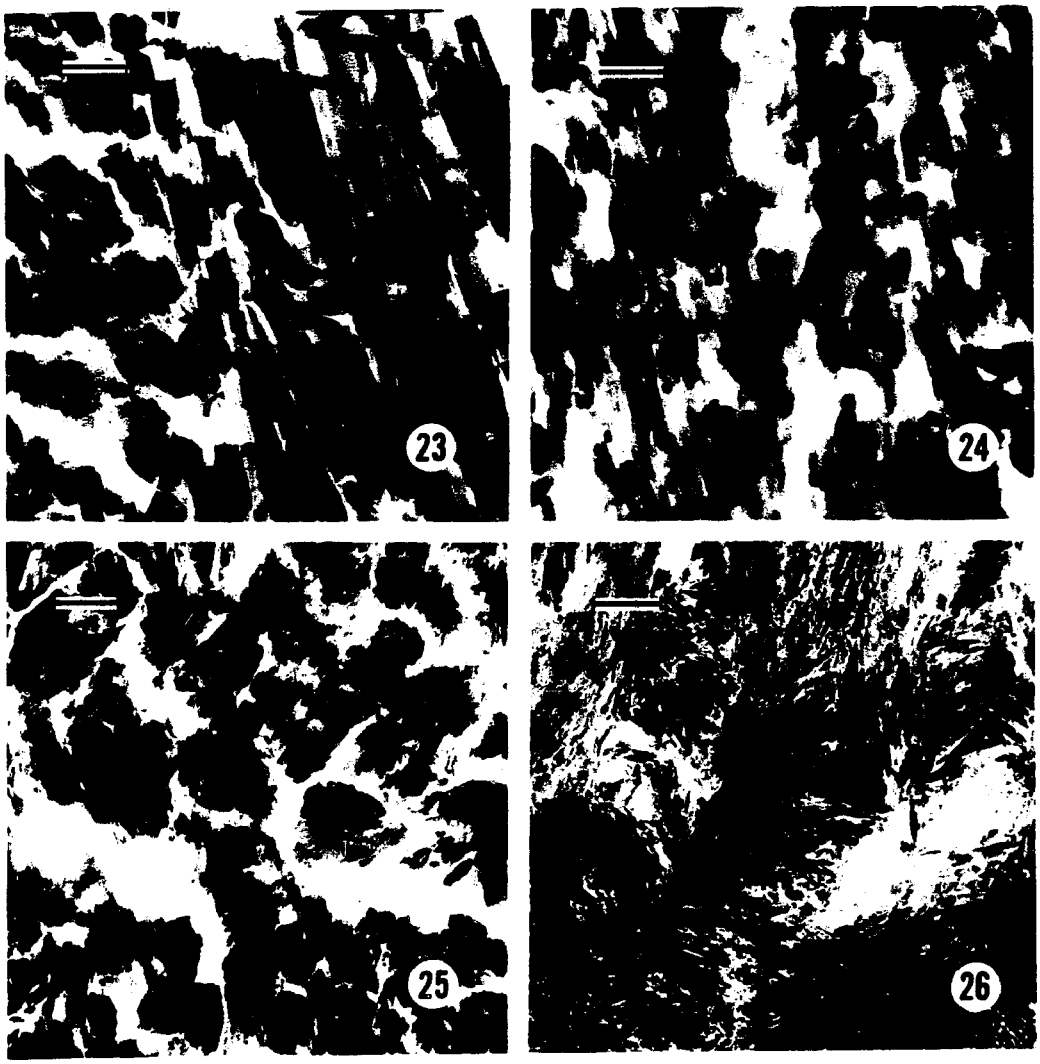
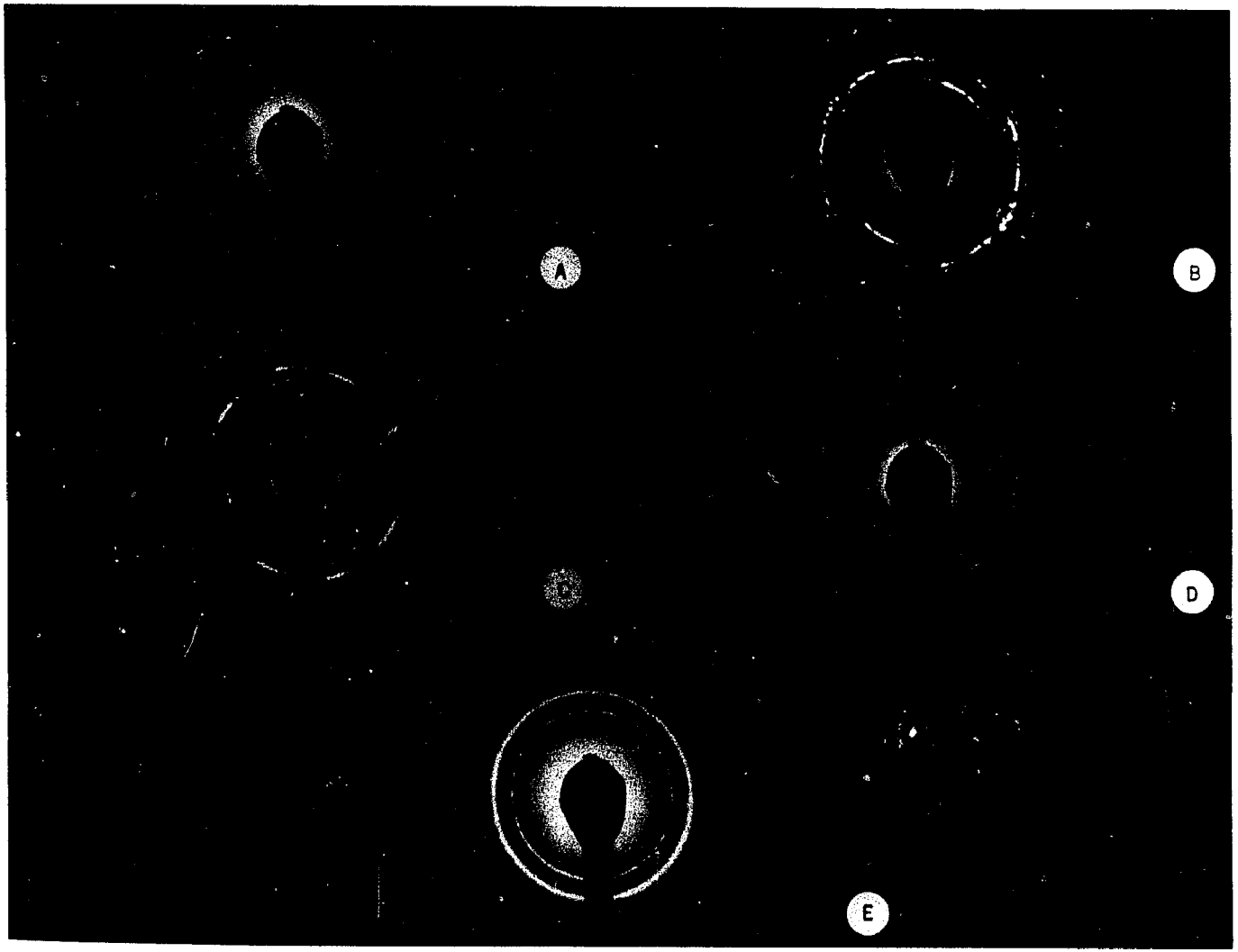

23
24
25
26

Figure 27.

Electron Diffraction Patterns of Selected Areas of the Enamel Surfaces.

Selected areas of about 1 μ in diameter 2 μ below the enamel surface.

A. Pattern of residual organic material after complete acid demineralization of the thin section of enamel on the grid.
B. Pattern of normal enamel.
C. Pattern of enamel immersed 14 hours in 2 mM sodium acetate buffer at pH 5.0.
D. Pattern of the mineral formed on the enamel surface after 4 days of immersion in a solution of 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0.05 mM [F] at pH 7.0.
E. Pattern of the material deposited on the enamel surfaces after precipitation of a solution initially containing 6.75 mM [Ca] and 4.05 mM [P] at pH 7.3.


A
B
C
D
E

solution. Figure 27E shows electron diffraction patterns of the materials precipitated on the enamel surfaces during immersion in the unstable solution of calcium and phosphate. The diffraction circles were spaced practically the same distances for the mineral samples, however, the granularity and the evenness of the circles differed between samples.

Figure 28 shows the SEM appearance at 1000 magnifications of the abraded surfaces of enamel before acid-etching. These pictures show the prismatic structure of enamel rods as they meet the surface as slightly depressed central regions of the rods.

Figure 29 shows the SEM appearance of acid-etched surfaces at 1000 magnifications. The central areas of the enamel rods dissolved while the peripheral areas of the enamel rods were retained. The depressions of the central regions and retention of the peripheral regions of the enamel rods produced a grid-like or honeycomb appearance on large areas of the etched surface. However, the acid-etch was not uniform over the entire surface.

Figure 30 shows the SEM appearance at 1000 magnifications of the acid-etched surfaces of enamel after 8 hour immersion in the metastable solution. The surface appears relatively smooth compared to the etched surface before immersion in the metastable solution. The honeycomb pattern persists in certain areas, as on the etched surface. However, the depressions in the central regions of the enamel rods appear smaller after the 8 hour immersion in the metastable solution.

Figure 28.

Scanning Electron Micrograph of a Lightly Abraded Surface of Bovine Enamel.

The bar indicates the length of 15 μ.

Figure 29.

Scanning Electron Micrograph of Enamel Surface Reacted with a Solution of 2 mM Sodium Acetate Buffer at pH 5.0.

The bar indicates the length of 15 μ.

Figure 30.

Scanning Electron Micrograph of Acid-Etched Enamel Surface After 8 Hour Reaction with a Metastable Calcifying Solution.

Etched surfaces of enamel were immersed 8 hours in a solution containing 1.5 mM [Ca], 0.9 mM [P], 150 mM [NaCl] and 0.05 mM [F] at pH 7.0. The bar indicates the length of 15 μ.

Figure 31.

Scanning Electron Micrograph of Acid-Etched Enamel Surface After 48 Hour Immersion in a Metastable Calcifying Solution.

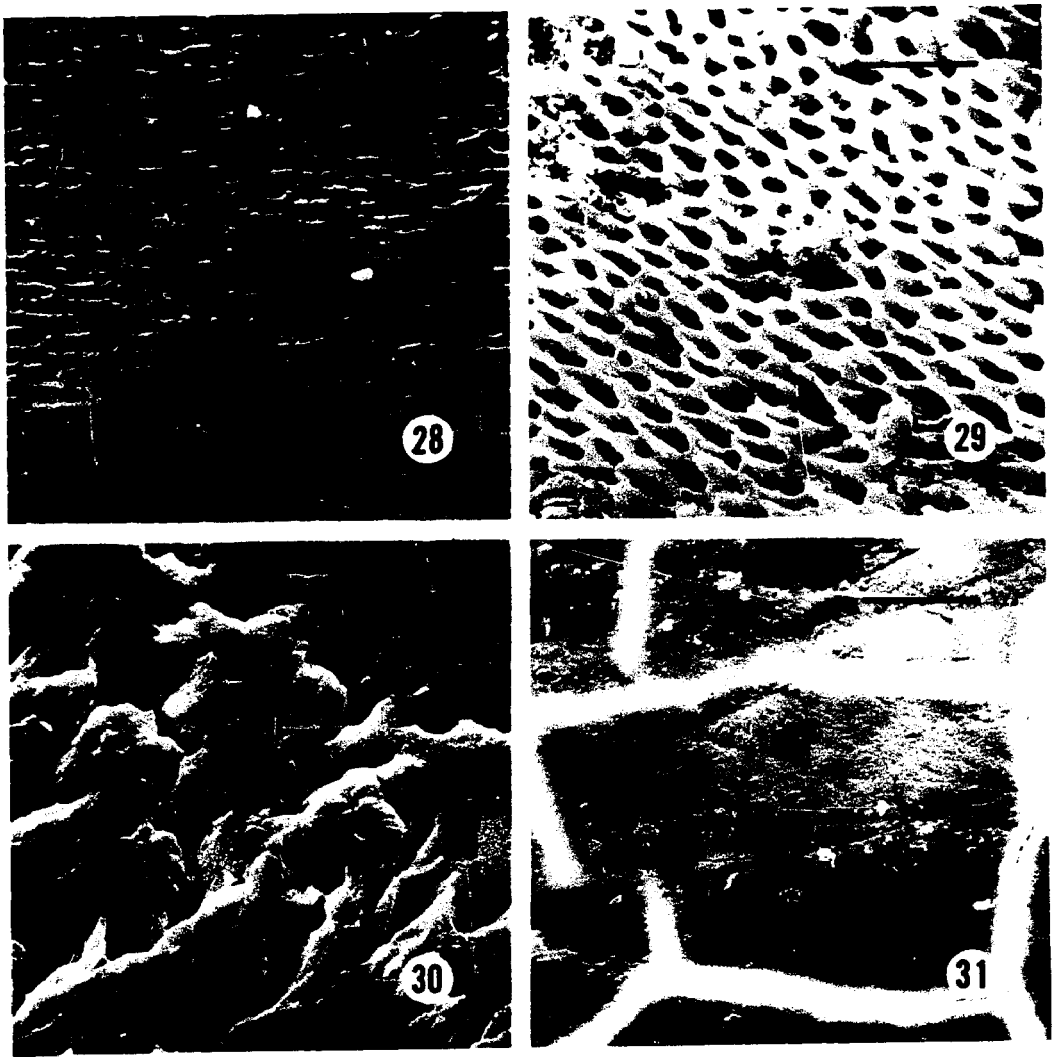

28
29
30
31

Figure 31 shows the SEM appearance at 1000 magnifications of the acid-etched surfaces of enamel after 48 hour immersion in the metastable solution. Except for the technique artifacts, i.e., cracks on the surface, the surfaces appear smooth and continuous, unlike the grid-like elevations and depressions on the acid-etched surfaces.

Discussion

The TEM and SEM pictures show areas of partial and complete dissolution of enamel tissue during the 14 hour immersion of the abraded enamel surfaces in the weak acid solution. The TEM showed enlargement of the intercrystalline spaces within the outer 10 microns of the labial surfaces with obvious greater acid reactions in the more central regions of the enamel prisms than in the peripheral regions of the prisms. The TEM showed less dense, partially dissolved, and loosely related crystals after acid-etching rather than the very dense tightly packed crystals of the normal enamel tissue. Similar TEM micrographs showing carious and acid-etched enamel crystals have been reported (Frank, 1967; Johnson, et al., 1971). TEM appearances in this study may differ somewhat with other TEM demonstrations of acid reactions of enamel crystallites because of the differences in acid strengths, volume to surface relationships, presence of diffusion retardants, directions of sectioning, thinness of sections, etc. It is

noteworthy that the acid reactions in this study occurred at fully mineralized surfaces of enamel before sectioning for TEM study.

The SEM showed preferential acid dissolution of the centers of the enamel prisms as did the TEM. In fact, both the TEM and SEM showed complete dissolution of the central regions of many prisms to depths of 5 to 10 microns. Since the apparent subsurface holes, seen in many of the TEM pictures, were filled with the araldite embedding material, they likely represent the completely dissolved central regions of the prisms sectioned diagonally rather than exactly perpendicular to the surface. The SEM appearance of the acid-etched enamel surfaces may resemble initial acid demineralizations in situ (Zuniga and Quigley, 1971). However, the acid-etching tremendously increased the total area of mineral surface which accounts for the greater rates of calcium phosphate mineral accretion at the acid-etched than the roughened and/or pumiced surfaces of intact enamel (Feagin, et al., 1972).

Approximately 70 to 76% of the Ca and 57% of the P dissolved from the enamel surfaces during acid-etching was deposited at the surfaces during 4 to 96 hour immersion in the metastable solution. The decreases of the calcium and phosphate concentrations in the metastable solution during the immersion of the enamel surfaces for several days, the stability of the solution without immersed enamel surfaces, and the greater rates of calcium and phosphate depletion in identical solutions with smaller volumes of solution and greater

areas of immersed surface, show calcium phosphate accretion at the enamel mineral surface.

The TEM and SEM studies show obvious mineral accretions within and on acid-etched surfaces of enamel after 24 hour immersion in the metastable solution. The SEM photographs show mineral accretions beginning at the central regions of the etched enamel prisms after the short time immersion (8 hour) in the metastable solution. Etched surfaces with completely dissolved prism regions were not found in any of the randomly selected surfaces after immersion in metastable solutions in the TEM studies. The SEM and TEM studies indicate mineral depositions which largely filled the surface irregularities resulting from the acid-etching.

The acid-etched areas of the surfaces appear slightly more electron dense after 4 and 8 hour mineral accretions. However, deposition of minerals in the intercrystalline spaces was not apparent in these short time reactions unless it occurred in the transition zone between the slightly etched crystals and the deeper unetched crystals. Microhardness studies of similar experiments indicate such mineral depositions in the transition zone between the deeper unetched minerals and the softened more superficial etched minerals (Feagin, et al., 1969).

The TEM studies clearly show the formation of a new crystalline mineral on enamel surfaces immersed in the metastable solution longer than 24 hours. The newly deposited mineral either completely replaced the enamel crystals or else deposited on unetched surfaces.

The new material may partly represent recrystallization of the etched enamel minerals into different crystal morphology during the lengthy immersions in the metastable solution. The morphology of new apatitic mineral was completely unlike the enamel mineral crystals although the electron diffraction patterns indicated similarity between the structures of enamel minerals and the newly deposited crystalline mineral. The electron diffraction patterns of the minerals deposited on the surfaces in metastable solutions show larger crystallite particle size than the particle size of the crystalline apatites precipitated on surfaces immersed in the unstable solutions. This was also apparent in the TEM appearance of the unprecipitated apatite and the minerals accreted on the enamel surfaces.

Summary

Reactions of mineral formation and dissolution occur on surfaces of enamel. The purpose of this study was to determine the E.M. appearances of acid-etched and "remineralized" enamel. Labial enamel surfaces, cut from bovine incisor teeth, were abraded and pumiced before immersion in 2 mM acetic acid of pH 5.0 for 14 hours. The etched surfaces were immersed in solutions containing 1.5 mM [Ca], 0.9 mM [P], 0.05 mM [F], and 150 mM [NaCl], and constant pH 7.0 and 34°C. Changes of Ca and P in the solutions were measured during mineral dissolution and deposition, and calculated for each

cm^2 of surface. The enamel samples were embedded (araldite) and 0.1 μ sections were cut perpendicular to the labial surface. T.E.M. pictures and electron diffraction patterns were obtained of the mineral surfaces of normal, acid-etched, and etched surfaces after calcium phosphate depositions. The acid-etched dissolved 31 μmoles/cm^2 calcium and 24 μmoles/cm^2 phosphate. Approximately 22.2 μmoles/cm^2 calcium and 13.6 μmoles/cm^2 phosphate deposited on the surfaces after 4 hour immersion in the solutions containing Ca and P. E.M. showed total and partial dissolution of enamel minerals 5 to 9 μ beneath the surface. The prismatic structure was retained in the etched enamel. The formation of mineral deposits of irregular crystallite shape were observed in the etched surfaces after immersion in the metastable solutions. The formation of the mineral deposits was most probably due to nucleation by partially demineralized crystals of enamel. Electron diffraction analyses showed that the mineral deposits were crystalline and had an apatitic structure.

CHAPTER V

CONCLUSIONS

From the results of this work, I conclude that:

The rates of interfacial reactions of labial enamel with metastable solutions of calcium and phosphate were faster during the initial 3 to 6 hours and, thereafter, approached chemical equilibrium as the calcium and phosphate concentrations decreased 60 to 70 percent.

The rates of reaction were greater in solutions containing 0.05 mM fluoride than those without fluoride.

Constant concentration of 0.05 mM fluoride in the calcium phosphate solutions caused fluoride ion incorporation and calcium enrichment in the minerals formed in the enamel-solution interface as compared to the products formed during reactions with solutions without fluoride.

The rates of fluoride ion incorporation into the enamel surface were constant in the pH range from 4.60 to 7.40 and were not affected by unrelated changes in the rate of reaction of calcium, phosphate or hydrogen ions.

Increase of the flow of solutions at the interface from 0.5 to 5 ml/min markedly increased the rates of mineral accretion by

removal of hydrogen ions released at the enamel surface as a consequence of calcium phosphate mineralization.

The density gradient distribution of powder scraped from the outer labial surfaces of pumiced bovine incisor teeth was 2.84 to 2.88 g/cm^3, both before and after mild etching in a dilute acid solution. The surface powder of enamel surfaces reacted 72 hours with metastable calcium phosphate solutions without fluoride equilibrated at a density range between 2.81 and 2.88 g/cm^3, and between 2.83 to 2.90 g/cm^3 after reactions with solutions containing 0.05 mM fluoride.

The close ranges of density demonstrate almost identical compositions in the powder samples scraped from untreated and experimentally altered enamel surfaces, with the possible exception of the small amounts of minerals formed from solution that did not contain fluoride.

Immersion of the enamel surfaces in weak acid for 14 hours caused partial and complete dissolution of the enamel mineral. Electron microscopy of the reacted surfaces showed enlargement of the intercrystalline spaces within the outer 10 μ of the labial surfaces with obviously greater acid dissolution in the more central regions of the enamel prisms. Acid demineralized crystals were less dense, partially dissolved and more loosely related than the tightly packed crystals of the normal enamel tissue. Electron and X-ray diffraction of the outer minerals of demineralized enamel showed no detectable alteration of the enamel crystalline structure.

Interfacial reactions of enamel with metastable solutions of calcium and phosphate caused the formation of deposits of irregular crystallite shape and electron density as observed in the electron microscope. The molecular mechanism of growth of the observed mineral deposit could be one of these processes: one, the formation of conglomerates of calcium, phosphate and hydroxyl or fluoride ions in the vicinity of preexisting crystals; or, two, the accretion of crystalline material on preexisting crystals of enamel. Both processes could account for the irregular morphology of the mineral deposited on the enamel surfaces. These deposits had larger crystallite particle sizes than the particle size of apatites precipitated on surfaces of enamel immersed in unstable calcium phosphate solutions. The crystallinity and apatitic nature of the mineral accretions were demonstrated with electron and X-ray diffraction.

BIBLIOGRAPHY

Angmar, B.; Carlström, D.; and Glas, J. E.: Studies on the Ultrastructure of Dental Enamel. IV. The Mineralization of Normal Human Enamel. J. Ultrastruc. Res. 8:12-23, 1963.

Armstrong, W.: Radiotracer Studies of Hard Tissues. Ann. N. Y. Acad. Sci. 60:670-684, 1955.

Bale, W.: A Comparative Roentgen-Ray Diffraction Study of Several Natural Apatites and the Apatite-Like Constituent of Bone and Tooth Substances. Amer. J. Roent. Radiol. Ther. 43:735-747, 1940.

Barker, L.; McPhillips, J.; Lawrence, G.; Doty, S.; Pallante, S.; Bills, C.; Scott, W.; and Howard, J.: Studies on Mechanisms of Calcification. Johns Hopkins Med. J. 127:2-22, 1970.

Baud, C. A.; and Lobjoie, D. D.: Biophysical Investigations on the Mineral Phase in the Superficial Layers of Human Dental Enamel. Helv. Odont. Acta 10:40-46, 1966.

Carlström, D.; Glas, J. E.; and Angmar, B.: Studies on the Ultrastructure of Dental Enamel. V. The State of Water in Human Enamel. J. Ultrastruc. Res. 8:24-29, 1963.

Chen, P. S.; Toribara, T. Y.; and Warner, H.: Microdetermination of Phosphorus. Analyt. Chem. 28:1756-1758, 1956.

Cocklica, V.; and Brudevold, F.: Density Fractions of Human Enamel. Arch. Oral Biol. 11:1261-1268, 1966.

de Jong, W.: La Substance Minerale dans les Os. Rec. Trav. Chim. 45:445-449, 1926.

Dirkes, O. B.: Posteruptive Changes in Dental Enamel. J. Dent. Res. 45:503-516, 1966.

Eanes, E.; and Posner, A.: Structure and Chemistry of Bone Mineral. In Biologic Calcification, Cellular and Molecular Aspects, ed. by H. Schraer. New York: Meredith Corp., 1970. pp. 1-26.

Feagin, F.: Calcium, Phosphate, and Fluoride Deposition on Enamel Surfaces. Calc. Tiss. Res. 8:154-164, 1971.

Feagin, F.; Gonzalez, M.; and Jeansonne, B.: Kinetic Reactions of Calcium, Phosphate and Fluoride Ions at the Enamel Surface-Solution Interface. Calc. Tiss. Res. 10:113-127, 1972.

Feagin, F.; Koulourides, T.; and Pigman, W.: The Characterization of Enamel Surface Demineralization, Remineralization and Associated Hardness Changes in Human and Bovine Material. Arch. Oral Biol. 14:1407-1417, 1969.

Feagin, F.; Walker, A.; and Pigman, W.: Evaluation of the Calcifying Characteristics of Biological Fluids and Inhibitors of Calcification. Calc. Tiss. Res. 4:231-244, 1969.

Fernandez-Moran, H.: A Diamond Knife for Ultrathin Sectioning. Exp. Cell Res. 5:255-256, 1953.

Frank, R.: The Ultrastructure of Tooth from the Point of View of Mineralization, Demineralization and Remineralization. Internat. Dent. J. 17:661-683, 1967.

Frazier, P. D.: Adult Human Enamel. II. An Electron Microscopic Study of the Effect of Grinding. Calc. Tiss. Res. 5:277-287, 1970.

Füredi-Milhofer, H.; Purgarić, B.; Brecevic, L.; and Pavkovic, N.: Precipitation of Calcium Phosphates from Electrolyte Solutions. I. A Study of the Precipitates in the Physiological pH Region. Calc. Tiss. Res. 8:142-153, 1971.

Gonzalez, M.; Jeansonne, B. G.; and Feagin, F. F.: Trimetaphosphate and Fluoride Actions on Mineralization at the Enamel-Solution Interface. J. Dent. Res. 52:261-266 (1973).

Head, S.: A Study on Saliva and Its Action on Tooth Enamel in Reference to Its Hardening and Softening. J. Amer. Med. Assoc. 59:2118-2122, 1912.

Higuchi, W.; Gray, J. A.; Henderson, J. J.; and Patel, P. R.: Mechanisms of Enamel Dissolution in Acid Buffers. J. Dent. Res. 44:330-341, 1965.

Hodge, H. C.: Hardness Tests on Teeth. J. Dent. Res. 15:271-279, 1936.

Jeansonne, B.; and Feagin, F.: Effects of Various Topical Fluorides on Subsequent Mineralization and Dissolution of Enamel. J. Dent. Res. 51:767-772, 1972.

Johansen, E.: Ultrastructural and Chemical Observations on Dental Caries. In Mechanisms of Hard Tissue Destruction, ed. by R. F. Sognnaes. Washington, D. C.: Amer. Assoc. Adv. Sci., 1963. pp. 187-211.

Johanson, B.: Remineralization of Slightly Etched Enamel. J. Dent. Res. 44:64-70, 1965.

Johnson, N. W.; Poole, D. F.; and Tyler, J. E.: Factor Affecting the Differential Dissolution of Human Enamel in Acid and EDTA. A Scanning Microscope Study. Arch. Oral Biol. 16:385-396, 1971.

Klug, H. P.; and Alexander, L. E.: X-ray Diffraction Procedures. New York: John Wiley and Sons, Inc., 1954. p. 504.

Koulourides, T.: Experimental Changes of Enamel Mineral Density. In Art and Science of Dental Caries Research, ed. by R. S. Harris. New York: Academic Press, Inc., 1968. p. 355.

Koulourides, T.; Cueto, H.; and Pigman, W.: Rehardening of Softened Enamel Surfaces of Teeth by Solutions of Calcium Phosphates. Nature 189:226-227, 1961.

Koulourides, T.; Feagin, F.; and Pigman, W.: Remineralization of Dental Enamel by Saliva in Vitro. Ann. N. Y. Acad. Sci. 131: 771-775, 1965.

Le Geros, R. Z.; Trautz, O. R.; Le Geros, J. P.; and Klein, E.: Infrared Studies of Biological and Synthetic Apatites. Abstract No. 62. In: Program and Abstracts of Internat. Assoc. for Dent. Res., San Francisco, 1968. Suppl. to J. Dent. Res., 1968b.

Lenz, H.: Ultrastructure of Tooth in Respect of Mineralization, Demineralization and Remineralization. Internat. Dent. J. 17: 693-708, 1967.

Little, M. F.; and Steadman, L. T.: Chemical and Physical Properties of Altered and Sound Enamel. IV. Trace Element Composition. Arch. Oral Biol. 11:273-278, 1966.

Luft, A. N.: Improvements in Epoxy Resin Embedding Methods. J. Biophys. Biochem. Cytol. 9:409-414, 1961.

Manly, R. S.; and Hodge, H. C.: Density and Refractive Index Studies of Dental Hard Tissues. I. Methods for Separation and Determination of Purity. J. Dent. Res. 18:133-141, 1939.

McCann, H. G.: The Solubility of Fluorapatite and its Relationship to that of Calcium Fluoride. Arch. Oral Biol. 13:987-1001, 1968.

Miller, W. D.: A Study of Certain Questions Relating to the Pathology of the Teeth. Dent. Cosmos 47:18-39, 1905.

Molnar, Z.: Development of the Parietal Bone of Young Mice. I. Crystals of Bone Mineral in Frozen-Dried Preparations. J. Ultrastruc. Res. 3:39-45, 1959.

Muhlemann, H.; Lenz, H.; and Rossinsky, K.: Electron Microscopic Appearance of Rehardened Enamel. Helv. Odont. Acta 8:108-111, 1964.

Nancollas, F.; and Mohan, M.: The Growth of Hydroxyapatite Crystals. Arch. Oral Biol. 15:731-745, 1970.

Naráy-Szabó, S.: The Structure of Apatite. Z. Kristallogr. Kristalgeon. 75:387-398, 1930.

Neuman, W.; and Neuman, M.: The Chemical Dynamics of Bone Mineral. Chicago: The University of Chicago Press, 1958.

Neuman, W.; Toribara, T.; and Mulryan, B.: Synthetic Hydroxyapatite Crystals. I. Sodium and Potassium Fixation. Arch. Biochem. Biophys. 98:384-390, 1962.

Posner, A.; Perloff, A.; and Diorio, A.: Refinement of the Hydroxyapatite Structure. Acta Crystallogr. 11:308-309, 1958.

Sarkar, B. C. R.; and Chauhan, U. P. S.: A New Method for Determining Microquantities of Calcium in Biologic Materials. Analyt. Biochem. 20:155-156, 1967.

Schraer, H.; Posner, A.; Schraer, R.; and Zipkin, I.: Effect of Fluoride on Bone Crystallinity in the Growing Rat. Biochem. Biophys. Acta (Amst.) 64:565-567, 1962.

Scott, D. B.; Simmelink, J.; and Nygaard, V.: Mineralization of Dental Enamel. In Symposium: Chemistry and Physiology of Enamel, ed. by The University of Michigan, Ann Arbor, 1971. pp. 6-24.

Silverstone, L. M.; and Johnson, N. W.: The Effect on Sound Human Enamel of Exposure to Calcifying Fluids in Vitro. Caries Res. 5:13, 1971.

Silverstone, L. M.; and Poole, D.: Histologic and Ultrastructural Features of Remineralized Carious Enamel. J. Dent. Res. 48: 766-770, 1969.

Sognnaes, R.: Microstructure and Histochemical Characterization of the Mineralized Tissues. Ann. N.Y. Acad. Sci. 60:545-574, 1955.

Speirs, R. L.: The Nature of Surface Enamel in Human Teeth. Calc. Tiss. Res. 8:1-16, 1971.

Stephan, R. M.: Intraoral Hydrogen Ion Concentration Associated with Dental Caries Activity. J. Dent. Res. 23:257-266, 1944.

Thomas, J. P.; and Feagin, F.: The Effects of 0.05 to 5.0 mM NaF on Calcium Phosphate Mineral Accretion and Subsequent Acid Dissolution at Enamel Surfaces. In Press, Ala. J. Med. Sci., 1973.

Volker, J. F.; Hodge, H.; Wilson, H. J.; and Van Voorhis, S. N.: The Adsorption of Fluorides by Enamel, Dentin, Bone and Hydroxyapatite as Shown by the Radioactive Isotope. J. Biol. Chem. 134:543-548, 1940.

Von der Fehn, F. R.: Maturation and Remineralization of Enamel. Adv. Fluor. Res. Dent. Caries Prev. 3:83-98, 1964.

Watson, M. L.; and Robinson, R. A.: Collagen-Crystal Relationships in Bone. II. Electron Microscope Study of Basic Calcium Phosphate Crystals. Amer. J. Anat. 93:25-59, 1953.

Weber, J. C.; and Posner, A.: X-ray Diffraction Assay of Enamel-Dentin Separation. Israel J. Med. Sci. 3:671-676, 1967.

Weidmann, S.; Weatherell, J.; and Hamm, S.: Variations of Enamel Density in Sections of Human Teeth. Arch. Oral Biol. 12:85-97, 1967.

Young, R.; and Spooner, S.: Neutron Diffraction Studies of Human Tooth Enamel. Arch. Oral Biol. 15:47-63, 1969.

Zipkin, I.: The Inorganic Composition of Bones and Teeth. In Biologic Calcification, Cellular and Molecular Aspects, ed. by H. Schraer. New York: Meredith Corp., 1970. pp. 60-103.

Zuniga, M.; and Koulourides, T.: Remineralization of Acid-Softened Human Enamel Studied by the SEM. Proceedings of the Fourth Annual Scanning Electron Microscope Symposium. In Scanning Electron Microscopy. Chicago: III Research Institute, 1971. pp. 361-368.

Zuniga, M.; and Quigley, M. B.: Incipient Carious Enamel Lesions Studied by Scanning Electron Microscopy. Proceedings of the Third Annual Scanning Electron Microscope Symposium. In Scanning Electron Microscopy. Chicago: III Research Institute, 1971. pp. 211-216.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Manuel Gonzalez Avila

Major Subject Physiology and Biophysics

Title of Dissertation Chemical Kinetics, Density and Electron Microscopy of Enamel Surface Reactions.

Dissertation Committee:

Frederick F. Feagin, Chairman
Marshall W. Hartley
Ward Pigman
T. Kowlowski
Edwin L. Bradley, Jr.
Charles E. Biggs

Director of Graduate Program Warren S. Pelham

Dean, UAB Graduate School S B Barker

Date 31 May 1973