Quantitative Microradiography for Studying Dental Enamel Demineralization and Remineralization

DAVID H. BERGSTROM *, JEFFREY L. FOX *, and WILLIAM I. HIGUCHI *x

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Abstract □ A method for quantitative determination of mineral density changes in dental enamel has been developed. It utilizes the quantitative microscopy capabilities of image analysis or photometer systems. Characteristic demineralization of bovine enamel in acetate buffers containing calcium and phosphate has been used to demonstrate the results. The method together with chemical kinetic data obtained from spectrophotometric and ion-selective electrode measurements of the bulk solution for calcium, phosphate, and fluoride permit important basic studies to be conducted on demineralization and remineralization of dental enamel.

Keyphrases □ Microradiography—quantitative study of dental enamel demineralization/remineralization □ Dental enamel—demineralization/remineralization, quantitative study using microradiography □ Demineralization—dental enamel, simultaneous remineralization, quantitative study using microradiography

Although the literature contains many references (1-5) utilizing microradiographic techniques to observe mineral changes that occur in enamel, the vast majority of these are largely qualitative in nature and are thereby limited in their usefulness. Until recent years, technology has limited the ability of researchers to obtain quantitative information from microradiographic images. The purpose of this present study was to develop and critically examine such a quantitative method and its limitations. The application of an image analysis system[1] to this quantitative determination of mineral density changes has been successful. Close examination of the density profiles obtained after incorporation of the internal standard data yields information not readily apparent from visual examination. The method is demonstrated here using demineralization of bovine tooth enamel, but it has also found application in synthetic hydroxyapatite compacts as well as examining the mineral density changes occurring in solutions containing fluoride.

EXPERIMENTAL

Dissolution Medium - A buffered solution ~32% saturated (on a molar basis) with respect to the thermodynamic solubility of hydroxyapatite was used for the demineralization procedure. The solution was a 0.1 M acetate buffer containing 7.05 mM each of total calcium and phosphate in the form of calcium chloride and dibasic sodium phosphate. The pH was adjusted to 4.5 with concentrated sodium hydroxide and the ionic strength to 0.5 M by the addition of sodium chloride.

Bovine Teeth Preparation—Incisors from 8-week old strictly Kosher calves were obtained from packing houses in the Chicago area. These are crate-fed calves whose diets are uniformly controlled and thus provide an excellent experimental system. From these, only reasonably flat teeth without any obvious surface defects were used experimentally. The pellicle was removed from the labial surface using 400-, and 600-grit silicon carbide[2] abrasive disks.

Dissolution Procedure—A bovine tooth was selected and covered with nail enamel, except for a 0.25 cm^2 square window on the labial surface. The tooth was then placed in 10 mL of 0.1 M acetate buffer containing 7.05 mM each of calcium and phosphate for varying lengths of time. It has been calculated that the dissolution medium is not saturated with respect to hydroxyapatite after these periods of dissolution. During demeneralization the solution and sample were gently agitated with a wrist-action shaker[3]. The temperature was maintained at 30°C throughout the procedure.

Sample Preparation for X-ray Analysis—The demineralized sample was mounted in a circular saw[4] with a high concentration diamond wafering blade.[5] Sections were cut through the exposed window perpendicular to the enamel surface as thin as possible. Each thin section was then ground with 600-grit silicon carbide paper plane-parallel to a thickness of 100 μm as determined by a micrometer. Final polishing using diamond paste[6] on a rayon cloth was done to remove any small aberrations resulting from the initial grinding procedure.

X-ray Method—The thin section of enamel was placed in direct contact with the emulsion of a 5 × 5 cm high-resolution glass plate[7]. A wedge made of bovine enamel was cut from the block enamel using the circular saw. The wedge was placed on the plate directly opposite the enamel surface to be analyzed. The internal standard is essential because it allows comparison of the negatives of different samples by factoring out any deviations due to variations in the time and temperature during the film development process. The sample was positioned in the center of the X-ray beam[8] for 25 min, with the instrument settings at 40 kVp and 3 mA. The exposed negative was developed in developer solution[9] according to the manufacturer's instructions. Quantitation of the mineral density *versus* position was accomplished using the image analysis system (Fig. 1). Scans were made through the demineralized region as well as the adjacent wedge (Fig. 2). The data were recorded on magnetic tape; a program[10] utilizing the wedge and sample data was employed to calculate the mineral density *versus* position.

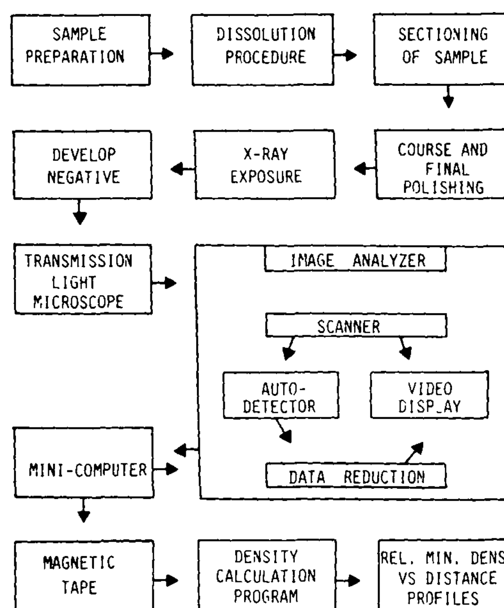

Figure 1—*Schematic representation of the methodology for obtaining relative mineral density* versus *position in enamel.*

[1] Quantimet-720, Cambridge Instruments, Inc., Monsey, N.Y.
[2] 3-M Co., St. Paul, Minn.
[3] Burrel Co., Pittsburgh, Pa.
[4] South Bay Technology, Temple City, Calif.
[5] Norton, Worcester, Mass.
[6] Mager Scientific, Inc., Ann Arbor, Mich.
[7] Type 1A; Eastman Kodak, Rochester, N.Y.
[8] Hewlett-Packard, McMinnville, Ore.
[9] D-19 Developer; Eastman Kodak, Rochester, N.Y.
[10] Developed by J. L. Fox, unpublished results.

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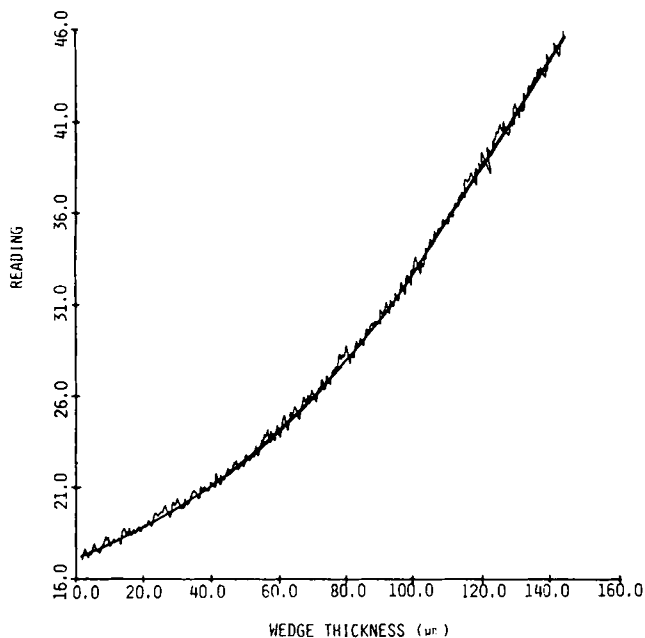

Figure 2—*Calibration plot using a wedge of enamel, providing the crucial link in allowing quantitative comparisons to be drawn between different microradiographs.*

RESULTS AND DISCUSSION

Contact Microradiography Technique—Contact microradiography, an extension of the conventional macroradiography, allows a rapid and convenient means of studying the density changes that occur within a block of enamel or a synthetic pellet (1) during the demineralization and/or remineralization process. This method possesses a very great depth of field and has the same resolution limit as transmitted light microscopy (~0.25 μm) (6). Several factors limit the resolution: (*a*) a lack of geometric sharpness results from the finite apparent source of the X-rays in relation to the source film distance; under the experimental procedure followed here this blurring of the image would be insignificant at ~0.02 μm as calculated by a previously described method (7); (*b*) high resolution glass plates have a photographic graininess limit of 0.15 μm; and (*c*) the lack of sharpness in the image at the edge of a sample resulting from the diffraction of the X-ray beam at the edge of a sharp electron-dense boundary. Again this effect was calculated to be ~0.2 μm and comparable to the film graininess (6). Considering all these contributions it can be assumed that structural detail down to ~0.5 μm can be observed. To test the resolution limit of the entire quantitative procedure, a scan was made over the sharp edge of a nondemineralized bovine tooth sample (Fig. 3), and the resolution limit was ~4 μm.

The system used to generate X-rays was operated at an accelerating voltage of 40 kVp and a current of 3 mA. The exposure time was 25 min, and the source-to-sample distance was 25 cm. A continuous spectrum of X-rays was produced, the wavelength of the most energetic being 0.05 nm. In an enamel sample the principal X-ray absorber at this wavelength is the calcium atom. The mass absorption coefficients of the inorganic *versus* organic components of enamel differ by a factor of 20. Absorption of X-rays of this wavelength by the organic material can be considered negligible.

The use of a wedge of bovine enamel exposed and further processed under the exact same conditions as the sample permits comparisons to be made between different negatives. Corrections can be thus made for any differences between negatives which may arise due to variations in time and temperature during the film development process. The angle of the wedge was determined by the trigonometric tangent function examining a negative on which the wedge resting on its side, had been exposed to X-rays.

Data Handling and Generation of Density Profiles—Using a computer program[10] the image data on the magnetic tape was transformed using the bovine wedge data as an internal standard to allow calculation and plotting of relative mineral density *versus* position.

In preparing a scan across the sample, the overall light level of the total image produced must be adjusted to allow maximum sensitivity of all the pixels (picture elements). The level of grayness can vary greatly between samples, so this adjustment must be made before each scan. When adjusting what is called white level sensitivity, the gray level values or scanner reading for the baseline value obtained from the exposed negative was not identical for two scans from the same negative (Fig. 3B *versus* Fig. 4B).

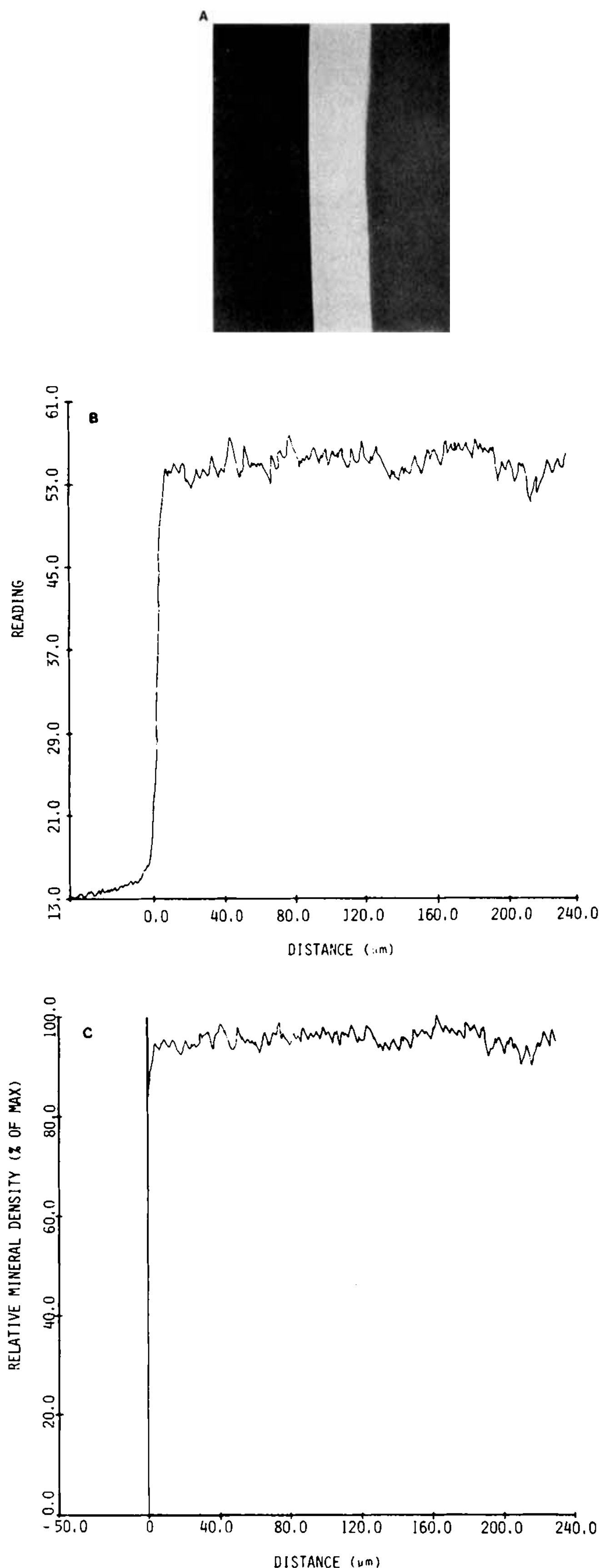

Figure 3—*(A) Photograph of a control sample of bovine enamel. Note the sharp edge between the exposed negative and the enamel. (B) Data obtained directly from the image analyzer after scanning from the negative onto the enamel to a depth of 230 μm. (C) Profile of relative mineral density* versus *position in enamel obtained after including the information obtained from the wedge data.*

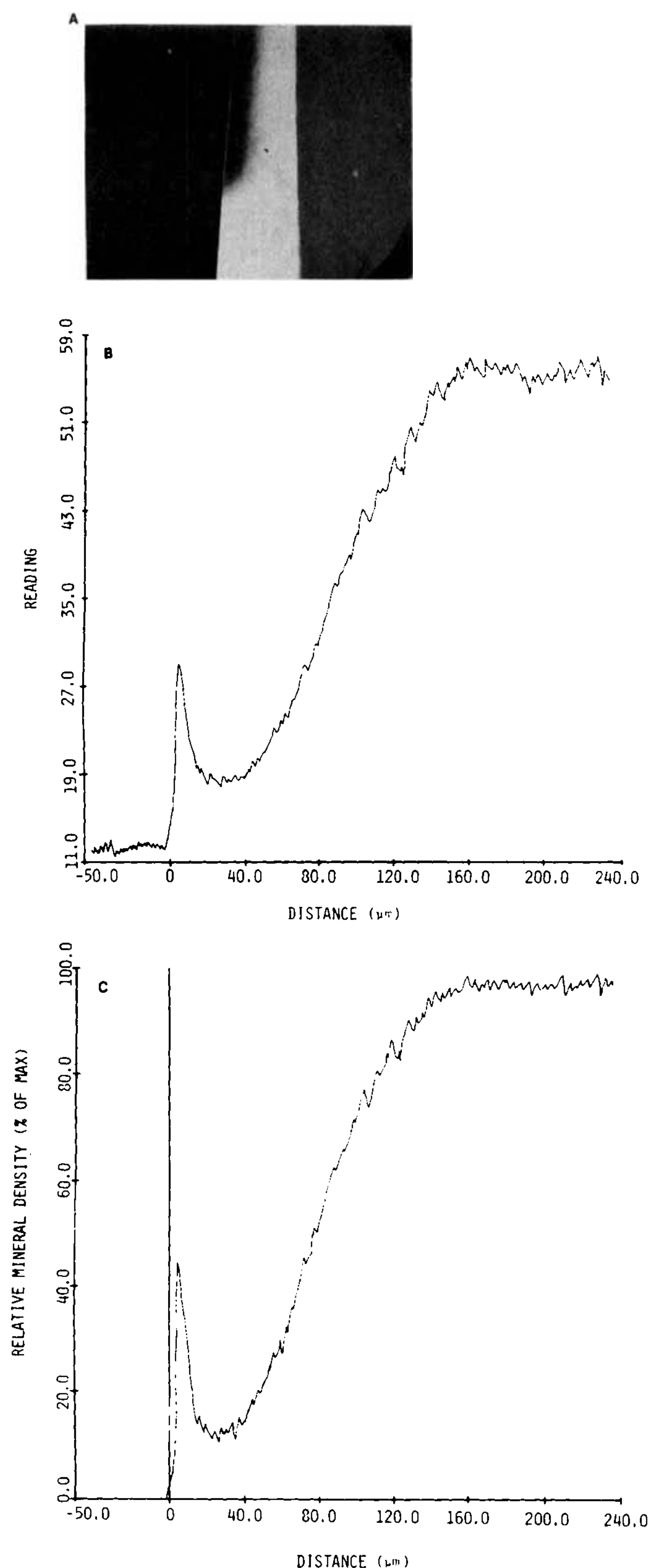

Figure 4—*(A) Photomicrograph of bovine enamel after 24 h demineralization at pH 4.5 in solution containing [calcium] = [phosphate] = 7.05 mM. (B) Image analyzer scanning of the microradiograph indicates mineralization of surface enamel is greater than that at depths further into the enamel. (C) Profile of mineral density* versus *position for (A) relative to the enamel at a depth of 230 µm.*

Initially it was thought that either the X-ray beam intensity or the photographic emulsion was not uniform, but scans made across different regions of blank negatives were essentially flat, indicating uniform exposure. To test

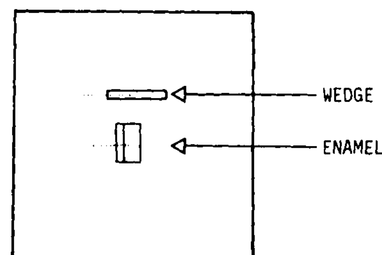

Figure 5—*Pictorial representation of microradiograph depicting relative positions of wedge and sample. The dotted lines show direction of image analysis scan.*

whether this variable baseline reading was due to this need to change the light intensity between samples, several scans across the same wedge in the exact same spot on the same negative were made. Results indicated that although the absolute values were changing they were changing at both ends of the curve so that the shape of the calibration curve remained constant. The placement of the wedge and sample (not to scale for clarification) is depicted in Fig. 5. The dotted line indicates the path each scan followed. These data allow normalizing the raw image analysis data obtained from the sample to that of the wedge and calculating from this the relative mineral density *versus* distance (see Figs. 2, 4B, and 4C).

The relative mineral density is defined as the normalized scanner reading at a point divided by the maximum reading in the sample × 100. The maximum scanner reading in the sample is taken as the average of the last 20 data points. In this it is assumed that there has been no acid demineralization in this deep recess of the enamel.

Application of the Method as Shown by Results of Experiments on Bovine Tooth Dissolution—In the development of this quantitative technique, samples of bovine enamel were exposed to a demineralizing solution for varying lengths of time (0, 1, 2, 3, 4, 5, 6, 12, and 24 h) and the resultant changes in mineral density were observed. For samples in which dissolution occurred for short periods of time, the mineral density was very low at the surface and increased monotonically until it reached the density of sound enamel. With increasing dissolution time, the mineral density at the original surface tended to increase, while the mineral beneath it remained less dense. The density again reached the maximum of sound enamel at sufficient depth (Fig. 4C). The longer dissolution times give rise to what has been termed a "subsurface lesion" or "white spot" phenomenon. This quantitative X-ray microradiographic technique has been shown to be an excellent method to study the demineralization and/or remineralization of enamel as well as synthetic hydroxyapatite compacts.

APPENDIX: Image Analysis

Image Analysis—Quantitative determination of the mineral density *versus* position in the tooth was obtained from the microradiograph using the image analysis system. This system is composed of three major components. The first is a transmission light microscope with high-quality optics, fitted with a 16× objective and 10× eye piece. The image analyzer itself is of modular design capable of a variety of image analysis functions, one of which is the image digitization used here. The third component of the system is a minicomputer system with a peripheral terminal and tape drive. The minicomputer functions here to collect and store the digitized image as it comes from the image analyzer and later to transfer this information to magnetic tape for further processing. A second function of the minicomputer is to instruct the system in its operations according to a user-defined protocol (average gray level determination) to be discussed later.

Image Production—The image analyzer uses a television camera to convert an optical image into an electronic signal. The first step then is to produce a high-quality image of the sample. The image analyzer cannot improve the quality of the projected image; in fact, its usefulness is severely limited by the quality of the image. Inherent in any image of good quality is lack of shading or uneven illumination in the image plane. Although an excellent grade microscope is used to produce the image, the optics are limited and image analysis off the optical axis of the microscope can result in incorrect measurements. In object plane scanning systems, *i.e.*, conventional microdensitometers, this problem is overcome by restricting the measurement area to a very small region in the center of the field on the optical axis of the microscope. In image plane scanners, *i.e.*, the system used herein, the measurement area is over a broad range, not limited to the optical axis of the microscope, so the resultant potential for error in off-axis operation must be corrected for. Fortunately, a unique comprehensive shading device has been developed which automatically corrects for illumination errors.

Glare or stray light arising from multiple reflections within the microscope

is another major source of error in microdensitometry, not unique to the image analysis method used here. Some of this stray light can affect the apparent optical density of the measured object. Glare tends to be specimen dependent, so its effect varies from field to field. In this system a dynamic glare correction is automatically applied. The transmission light microscope is the device utilized to produce an optical image which is then projected onto the photosensitive face of the vidicon tube scanner[11].

Image Scanning—The face of the vidicon tube is made of material that produces a local potential difference proportional to the intensity of light striking it. The sampling method of the scanner then converts the optical image to an electronic signal consisting of continuously varying analog voltage. High voltage corresponds to bright (white) portions of the image, while low voltage corresponds to the darker regions.

Image Digitization—The total image is composed of ~500,000 picture points or pixels, each of which is sensitive to the light intensity incident on it and contributes to the total analog video signal. It is within the auto detector module (see Fig. 1) that the analog video signal of each pixel is assigned a "gray level" value between 0 and 63. The 63 value corresponds to the brightest area over the total image, the 0 value set to some internal standard for black. The result of this digitization procedure is a digital image consisting of a matrix of numbers each with a value between 0 and 63. This is the basic data set produced by the image analysis system.

Data Reduction Module—To allow selectivity as to what portion of the total field is to be scanned, a variable frame size option is employed. This allows restricting the measurement to some quadrilaterally defined portion of the field. A frame size measuring 600 pixels horizontally by 10 pixels vertically is used to scan each sample. The 600 horizontal pixels under the 160× magnification of the microradiograph allows a sufficiently wide scan to be made across the sample and adjacent negative.

The minicomputer interface allows further processing of the 600 × 10 matrix of pixels, each with a value between 0 and 63. In this case, it is the 10 vertical pixels that are acted upon. To minimize any error due to variable sensitivity of a particular pixel, some defect in the microradiograph itself (dust or a scratch), a single value is obtained from the 10 vertical numbers by simply taking the average. This produces what is called an "average gray level" for 600 horizontal sampling points. Using appropriate calibration methods it was determined that under these conditions each pixel has a width of 0.46 μm. The data are then transferred to magnetic tape as a pair of numbers corresponding to position in scan and the average gray level or "reading" at that position.

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Quantitative Microradiographic Study of Simultaneous Demineralization/Remineralization of Dental Enamel in Weak Acid Buffers

DAVID H. BERGSTROM *, JEFFREY L. FOX *, and WILLIAM I. HIGUCHI *x

Received May 17, 1982, from *the Dental Research Institute and the College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109.* Accepted for publication March 7, 1983. *Present address: Department of Pharmaceutics, College of Pharmacy, The University of Utah, Salt Lake City, UT 84112.

Abstract □ The remineralization behavior of weak acid-treated bovine tooth enamel has been investigated using a recently developed quantitative microradiographic method. Acetate buffer solutions at pH 4.5 containing calcium, phosphate, and 10 ppm fluoride were used in this study. When the solution ion activity product ($K_{FAP} = a_{CA}^{10} a_{PO_4}^{6} a_{F}^{2}$) was 1×10^{-108}, the remineralization of the demineralized region was relatively uniform and complete. On the other hand, when the K_{FAP} was ~ ≤1 × 10^{-112}, remineralization of the outer 10-20 μm was incomplete. In addition, for the smaller K_{FAP} solutions there was significant demineralization in the deeper recesses of the originally demineralized region. These results agree with a recent chemical kinetics study in which it was proposed that $K_{FAP} = 1 \times 10^{-112}$ demarcated the region of solution conditions in which remineralization only occurs from that in which simultaneous demineralization/remineralization takes place. A model consistent with all of the data is proposed.

Keyphrases □ Microradiography—quantitative study of dental enamel demineralization/remineralization, solution ion activity product □ Dental enamel—demineralization/remineralization, solution ion activity product, quantitative microradiography □ Demineralization—dental enamel, simultaneous remineralization, solution ion activity product, quantitative microradiography

Recently, Fox *et al.* (1) investigated the influence of the solution ion activity product ($K_{FAP} = a_{Ca}^{10} a_{PO_4}^{6} a_{F}^{2}$) on the remineralization of weak acid-treated bovine tooth enamel and hydroxyapatite pellets. Solutions containing calcium-45, phosphate, and fluoride in acetate buffers were used. The calcium-45/fluoride molar ratios determined for the remineralized enamel by perchloric acid etch biopsy indicated the predominant formation of fluorapatite [$Ca_{10}(PO_4)_6F_2$; FAP] or fluoridated hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_{2-x}F_x$], where $0 \leq x \leq 2$ when $pK_{FAP} \simeq 108$. When the pK_{FAP} of the solutions were ≥112, the calcium-45/fluoride ratios were found to be considerably <5. From these results, the authors proposed that a pK_{FAP} value of ~112 marked the demarcation between remineralization only and simultaneous demineralization/remineralization for these acidic remineralizing solutions.

The purpose of the present investigation was to study the remineralization of bovine tooth enamel under the same conditions as those of Fox *et al.* (1), employing a recently developed (2) quantitative microradiographic technique. A special point of interest was to examine the hypothesis that simultaneous demineralization/remineralization sets in when the pK_{FAP} value is ~ ≥112 for these solutions.

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