

SECRETORY ACTIVITY AS A FUNCTION OF THE DEVELOPMENT AND MATURATION OF AMELOBLASTS.

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Abstract The biosynthetic and secretory activity of rat incisor ameloblasts was studied by grain count analysis of radioautographs at various times following a single injection of either ^3H -methionine, ^3H -leucine, or ^3H -glycine. Experiments were also carried out with leupeptin, a thiol and serine proteinase inhibitor which blocks degradation of proteins within lysosomes. The results from this study indicate that the biosynthetic and secretory activities of ameloblasts increase steadily as the cells differentiate (presecretory stage) and start to form the enamel layer (secretory stage). Secretory activity reaches a peak when the ameloblasts form about one-third of the eventual thickness of the enamel, and remains at this high level until shortly before they start to form the outer and final layers of enamel. Secretory activity then drops rapidly as the cells undergo postsecretory transition, and declines slowly thereafter as the shortened ameloblasts modulate continuously along the surface of the maturing enamel. Ameloblasts appear to biosynthesize more proteins than are secreted. The excess proteins are degraded rapidly in lysosomes and the amino acids reutilized for production of new exportable and/or structural proteins.

INTRODUCTION

It is widely believed that ameloblasts acquire the organelles and "information" necessary for production of enamel proteins only just before, or at the time when, they complete their differentiation sequence at the surface of the mineralizing mantle preentin^{1,2}. It is also generally accepted that ameloblasts stop forming enamel matrix components when they undergo postsecretory transition^{1,2}. Recent studies suggest, however, that ameloblasts secrete small amounts, and varying types, of enamel proteins, and other exportable proteins, as they differentiate (presecretory stage)^{3,4} as well as while they modulate (maturation stage)^{4,5}.

Although much basic information is presently available about the biosynthesis and intracellular transport of enamel proteins¹⁻⁶, few workers have attempted to quantify changes in the absolute level of secretory activity that occur sequentially over time as ameloblasts differentiate, form the enamel layer, and then alternate between ruffle-ended and smooth-ended morphologies along the maturing enamel surface. The purpose of this study was to characterize these changes using radioautographs of rat incisors and computer image processing to assist grain counting analyses at consecutive 250 μm intervals along the entire length of these teeth.

MATERIALS & METHODS

Fifteen male Wistar rats weighing about 100 g were anesthetized with sodium pentobarbital (0.06 ml/100 g b.w.) and injected via the external jugular vein with 1 mCi of either ^3H -methionine (s.a., 80 Ci/mmol), ^3H -leucine (s.a., 147 Ci/mmol), or ^3H -glycine (s.a., 53 Ci/mmol) suspended in 0.1 ml of 0.05 M PBS (pH 7.4). After 6 min, each rat was then given an intravenous cold chase with 0.1 ml of a 10 mM solution of the same non-radioactive L-amino acid suspended in 0.05 M PBS (pH 7.4). The animals were killed in pairs, or groups of three, at various intervals from 10 min to 8 hr after the radioactive injection by vascular perfusion with either 4% paraformaldehyde or 5% glutaraldehyde⁵. In one experiment (Fig. 7), rats first received a single intraperitoneal injection of leupeptin (30 mg/100 g b.w.) suspended in 0.05 M PBS two hr prior to injection of ^3H -methionine. These animals were given a cold chase 6 min after the ^3H -methionine and then killed at 1 hr from the radioactive injection. Following perfusion fixation, the mandibles were removed from each rat, decalcified in EDTA, and the incisors were split into segments and processed for embedding in Epon. The entire length of each incisor was reconstructed for light microscopic radioautography by cutting 1- μm -thick sagittal sections from one of the blocks from each of the 4 segments into which a given tooth was divided. The sections were prestained with hematoxylin, dipped in liquid emulsion, exposed for 14 days and developed^{4,6}.

The distribution of silver grains over ameloblasts and developing enamel was quantified on a microcomputer system within a counting window having dimensions of 10 μm (height) by 20 μm (width)⁶. The height of the window could be adjusted to allow for counting over histological structures that filled only a portion of the window. On each incisor, the top of the window was first aligned with the apex of ameloblasts at the start of the region of ameloblasts facing dentin (region "PS" on the abscissa of the graphs in Figs. 1-3, 5, 7)⁷. The number of picture elements (pixels) that represented silver grains in the digitized image was counted in this window as well as in adjacent, but non-overlapping, windows vertically across the thickness (height) of the ameloblasts layer. The microscopic field was then advanced 250 μm in an incisal direction (tic interval along abscissa of the graphs in Figs. 1-7), and grain counts were made within a series of windows traversing the thickness of the ameloblast layer at this site. This process was repeated at consecutive 250 μm intervals along the entire length of the incisor up to the gingival margin. At sites where enamel was present at the apex of ameloblasts (positions "SEC" to "ES", Figs. 1-7), grain counts were made first over ameloblasts as described above and then the bottom of the window was aligned with the apex of ameloblasts and grain counts were made vertically across the thickness of the enamel layer to the dentinoenamel junction. Within the the secretory zone (positions "SEC" to "MAT", Figs. 1-7), any silver grains over the interdigitating portion of

Tomes' processes were counted as part of the enamel. Grain counts were made in a similar fashion for all mandibular incisors in a group (4-6 incisors per amino acid per time interval). Data for summed grain counts at each 250 μm interval were averaged across all teeth for a given amino acid and time after injection and expressed as the mean number of silver grains (pixels) over ameloblasts (Fig. 2) or over enamel (Figs. 4, 6, 8). The density of labeling over ameloblasts at each 250 μm interval along the length of the incisor (Figs. 2,3,5,7) was computed by dividing the the mean number of silver grains (pixels) over ameloblasts at each interval by the mean number of windows required to count across the ameloblasts at this site. Profile height, or thickness, of the ameloblast and enamel layers (Fig. 1) was deduced from the window data (e.g., 5.4 windows = 54 μm). While not shown in Figures 1-7, the coefficient of variation rarely exceed 25% of the mean for grain counts over ameloblasts or enamel. Background labeling was less than 35 pixels out of a total of 34,606 possible pixels per window⁶. No correction of raw data (Figs. 2-7) was made for this very low background within the radioautographs.

RESULTS

Quantitative analyses at consecutive 250 μm intervals along the length of mandibular rat incisors indicated that both the height of ameloblasts and the thickness of the enamel layer changed dramatically over the course of amelogenesis (Fig. 1). In the case of ameloblasts, this included (a) a rapid increase in height from 35 μm to about 54 μm between the beginning and end of their initial differentiation across the presecretory stage, (b) a gradual increase in height to a peak value of about 70 μm halfway across the secretory stage of amelogenesis, (c) a gradual decrease in height from 70 μm to about 58 μm during formation of the outer and final layers of enamel, and (d) an abrupt decrease in height from 54 μm to about 40 μm during postsecretory transition (Fig. 1). Ameloblasts changed little in height thereafter until they became reduced (20-10 μm) near the gingival margin (not shown in Fig. 1). In contrast, the enamel layer increased linearly in thickness across all but the first 250 μm of the secretory stage reaching a maximum of about 100 μm by the beginning of maturation stage of amelogenesis (Fig. 1). The enamel layer then decreased gradually in thickness to about 84 μm near the site where it was completely soluble in EDTA (Fig. 1, "ES"). Regression analysis indicated that the thickness of the enamel layer increased at an average linear rate of 13.7 μm per day across the secretory stage of amelogenesis⁸ (Fig. 1; correlation coefficient = 0.9991).

Grain counts revealed characteristic regional differences in the amount of radioactivity incorporated by ameloblasts at 10 min after injection of ³H-methionine (Fig. 2). In broad terms, secretory stage ameloblasts showed the highest labeling and mid to late matura-

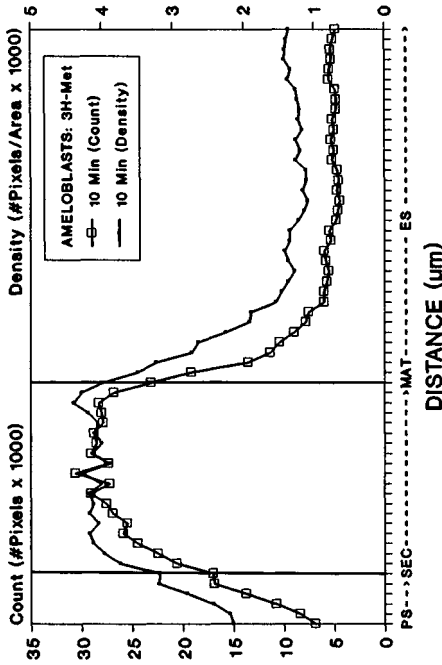


FIGURE 2. Number of silver grains over ameloblasts at 10 min after injection of 3H-methionine (left axis). Data is also expressed as density of labeling (right axis). See legend to Figure 1 for explanation of abscissa.

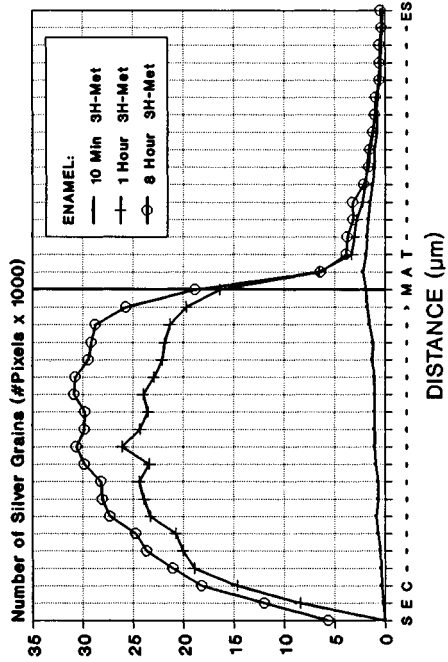


FIGURE 4. Number of silver grains over enamel at 10 min, 1 hr and 8 hr after injection of 3H-methionine. Note that the abscissa has been expanded relative to Figure 3. Only the portion of the tooth from the start of the secretory stage (SEC) to the EDTA soluble area in the maturation stage (ES) is shown.

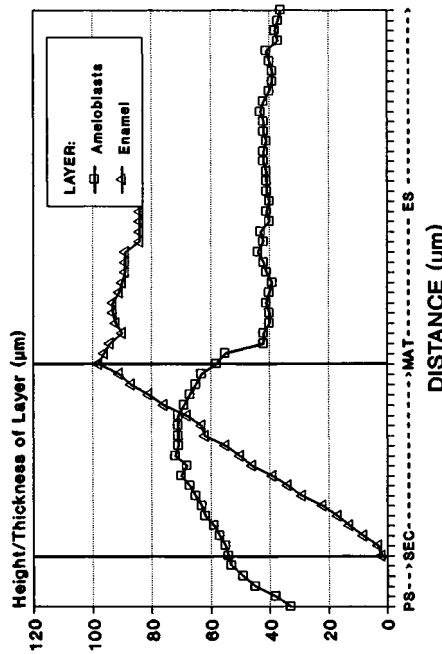


FIGURE 1. Ameloblast height and enamel thickness as measured at consecutive 250 µm intervals across the incisor. Vertical lines demarcate the boundaries of the secretory (SEC) stage of amelogenesis. PS, presecretory stage; MAT, maturation stage; ES, site where enamel is soluble in EDTA.

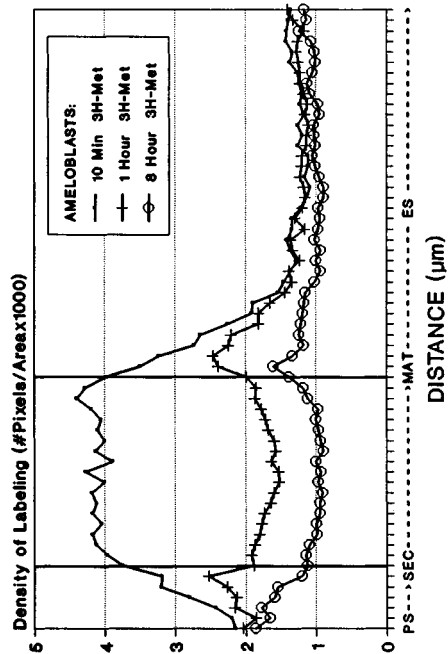


FIGURE 3. Density of labeling over ameloblasts at 10 min, 1 hr and 8 hr after injection of 3H-methionine. See legend to Figure 1 for explanation of abscissa.

tion stage ameloblasts the lowest labeling at 10 min (Fig. 2). Area corrected data indicated that the density of labeling over ameloblasts increased about two-fold progressively over the length of tooth where ameloblasts underwent differentiation and formed the first 20 μm of the enamel layer (Fig. 2). The density of labeling over ameloblasts remained high across the rest of the secretory stage, then dropped steadily to a four-fold lower level by the mid maturation stage of amelogenesis (Fig. 2, "ES"). At 10 min after injection, the enamel showed a weak labeling with a small peak of radioactivity near the region of postsecretory transition (Fig. 4, "MAT").

From 10 min to 1 hr after injection, there was a profound drop in the density of labeling over secretory stage ameloblasts and those located in the region of postsecretory transition (Fig. 3). The density of labeling over presecretory stage ameloblasts also decreased, but little, or no, change in the density of labeling was evident over mid and late maturation stage ameloblasts (Fig. 3). These changes resulted in the creation of two peaks of labeling, one located near the boundary between presecretory and secretory stages and the other located near the boundary between the secretory and maturation stages of amelogenesis (Fig. 3). Similar peaks of labeling were also evident at 1 hr in animals injected with ^3H -leucine but not in those injected with ^3H -glycine (Fig. 5). The peaks of labeling were also greatly exaggerated in animals which were injected with leupeptin prior to ^3H -methionine (Fig. 7). The enamel was heavily labeled by 1 hr in animals injected with ^3H -methionine (Fig. 4) and ^3H -leucine (Fig. 6). There was a gradient in labeling with progressively more silver grains over enamel from the beginning up to about the middle of the secretory stage of amelogenesis. There was a decrease in the number of silver grains over enamel near the end of the secretory stage followed by a sharp drop in labeling over enamel in the region of postsecretory transition and a gradual decline thereafter up to the site where enamel was soluble in EDTA (Fig. 4, "ES"). Animals injected with leupeptin showed a normal labeling over enamel at 1 hr after injection of ^3H -methionine (Fig. 8).

The density of labeling over late presecretory, secretory and early maturation stage ameloblasts continued to decline from 1 hr to 8 hr after injection (Fig. 3). Secretory stage ameloblasts and most maturation stage ameloblasts showed the same density of labeling by 8 hr (Fig. 3). There was, however, a slight peak of higher labeling over ameloblasts in the region of postsecretory transition and a noticeably higher labeling over young differentiating ameloblasts at this time (Fig. 3). The number of silver grains over enamel increased from 1 hr to 8 hr especially in relation to secretory stage enamel and to regions near the boundary between inner and outer enamel secretion (Fig. 4). The peak observed for secretory stage enamel at this time corresponded roughly to the site of maximum labeling in ameloblasts at 10 min after injection (Figs. 2 & 4).

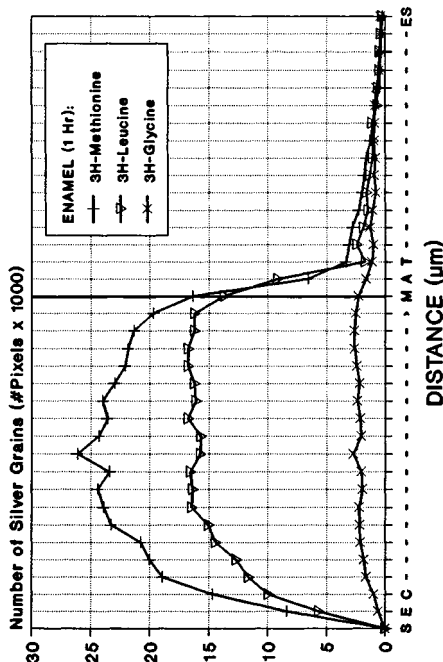


FIGURE 5. Density of labeling over ameloblasts at 1 hr after injection of 3H-methionine, 3H-leucine, and 3H-glycine. See legend to Figure 1 for explanation of abscissa.

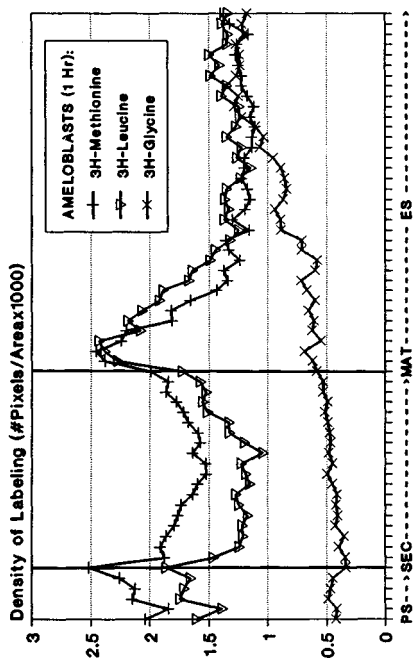


FIGURE 6. Number of silver grains over enamel 1 hr after injection of 3H-methionine, 3H-leucine and 3H-glycine. Note that the abscissa has been expanded relative to Figure 5. Only the portion of the tooth from the start of the secretory stage (SEC) to the EDTA soluble area (ES) is shown.

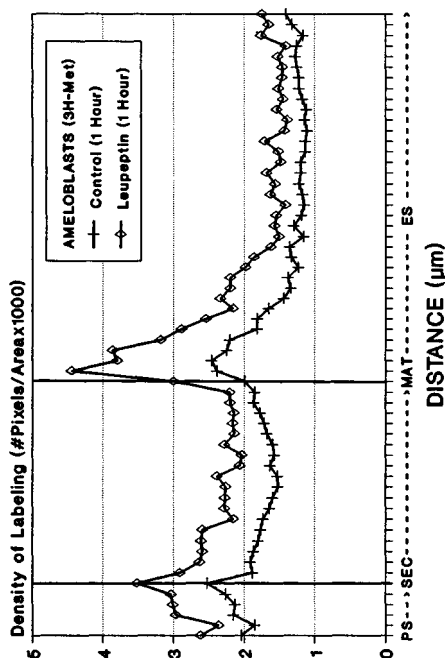


FIGURE 7. Density of labeling over control and leupeptin-treated ameloblasts at 1 hr after injection of 3H-methionine. See legend to Figure 1 for explanation of abscissa.

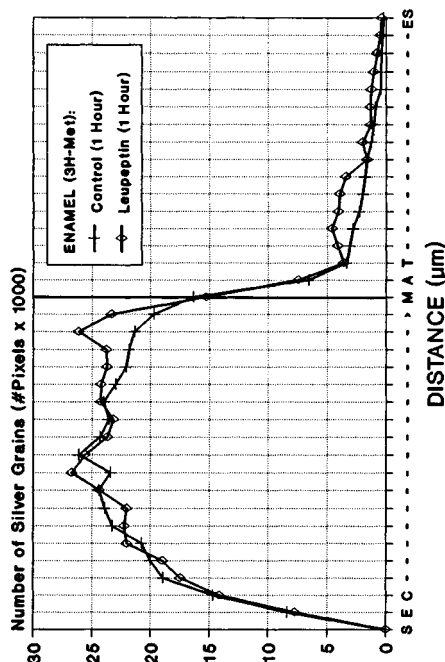


FIGURE 8. Number of silver grains over enamel in control and leupeptin-treated rats at 1 hr after injection of 3H-methionine. Note that the abscissa has been expanded relative to Figure 7. Only the portion of the tooth from the start of the secretory stage (SEC) to the EDTA soluble area (ES) is shown.

DISCUSSION

The results of this study provide a simple demonstration of the power of computer image processing in allowing quantitative experiments to be designed which otherwise would have been impossible by older grain counting techniques. The importance of this approach of quantifying incisors at regular intervals across all stages of amelogenesis is best illustrated in Figures 3, 5 and 7 where subtle changes in the regional distribution of silver grains over a few hundred micrometers of tooth length would likely have been missed if random, or spot zonal, sampling of the mandibular rat incisors had been employed.

Although it has been known for many years that the secretory activity of ameloblasts is greatest over the period of time during their life cycle when they form the enamel layer by appositional growth¹⁻⁸, it has not been obvious that there are changes in the height of the cell, in the amount of radioactivity incorporated immediately after injection of radiolabeled amino acids, and in the amount of radioactive proteins secreted by the cell over 8 hr, depending upon whether the ameloblasts are forming the initial, inner, outer, or final layers of the enamel. Similarly, it has not been evident that ameloblasts located in the region of postsecretory transition at the time of injection secrete almost as much radioactive material as those ameloblasts which form the initial layer of enamel by 8 hr after injection (Fig. 4, "SEC" and "MAT"). Of interest in Figures 2-4 was the finding of an apparent fast (10 min to 1 hr) and slow (1 hr to 8 hr) components to the movement of radiolabeled proteins from ameloblasts into enamel. It is also curious that the total amount of radioactivity transferred from ameloblasts to enamel by 8 hr during the secretory stage of amelogenesis was about equal to the absolute amount present in these cells at 10 min after injection at all sites except near the very beginning and end of this stage where the enamel gained only between 35-80% of the total amount of radioactivity initially incorporated by the overlying ameloblasts at 10 min (Figs. 2 & 4).

The results of the experiment with leupeptin provide some insight into the possible mechanism by which these phenomena may arise. That is, leupeptin-treated ameloblasts clearly secrete normal amounts of radioactive proteins into the developing enamel (Fig. 8) but they contain higher than normal amounts of radioactivity intracellularly at 1 hr after injection. Since leupeptin blocks degradation of proteins in lysosomes⁹, a likely explanation for the high counts over ameloblasts is that these are due to the presence of newly formed, radioactive proteins in lysosomes that otherwise would have been degraded over the 1 hr interval from the initial injection of the precursor amino acid. Hence, the first component in the movement of radioactive proteins from ameloblasts to enamel observed up to 1 hr (Fig. 4) represents secretion of proteins biosynthesized in a "wave" relative to the initial injection of the amino acid. The second component in the movement of radioactive proteins

from ameloblasts to enamel observed beyond 1 hr (Fig. 4) seems to be due to reutilization of amino acids derived from lysosomal degradation of other labeled proteins that were formed in the first wave, but not secreted, for the production of new proteins that are produced in later waves. Data from this study suggests that the amount of proteins cycling through the lysosomal system could represent as much as 10%, or more, of the total quantity of exportable proteins being formed by ameloblasts at any given point in time. This is consistent with amounts reported for other cells, including fibroblasts, where newly synthesized proteins are degraded continuously in lysosomes¹⁰.

Variations in the amount of protein routed to lysosomes could also provide a mechanism by which ameloblasts exert fine control over the quantity of proteins secreted at specific times during amelogenesis. While it could be argued that the increase and decrease in the labeling curve observed for ³H-methionine in Figure 4 are due to differences in amino acid composition (types), and not quantity, of proteins being produced, the findings of identical changes in the labeling curves for two other amino acids (Fig. 6) and high sensitivity of ameloblasts to leupeptin in areas where the labeling curves are below peak levels (Fig. 7) suggest that these represent real quantitative difference and that proportionately more newly formed proteins undergo lysosomal degradation at some sites (e.g., postsecretory transition) compared to others (mid secretory stage). Hence, the lysosomal system may provide the ameloblast with a method to partially regulate the flow of exportable proteins in a cell which otherwise appears to secrete proteins constitutively¹¹.

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QUESTIONS FOR DR. SMITH

H. Slavkin: If I'm following correctly, you are suggesting that within the secretory ameloblasts there might be two pathways that synthesize material that might be shunted to lysosomes; and they may either just sit there and therefore account for the continued high counts or they might be recycled?

C. Smith: Yes, and we think that the latter case may be the correct or more common pathway. Don't forget that these animals are under a condition of an extreme cold chase - we injected a large quantity of cold amino acids, and we can't chase them out of the system very quickly. So I believe we have evidence of several cell types, one over-producing secretory material and one conserving amino acids that are being broken down in the lysosomes.

S. Sasaki: How do you explain the glycine curve? It looked very strange.

C. Smith: Yes, we were very surprised. Well it is based on the current literature. It is fairly well accepted that the amelogenins are glycine poor, and traditionally the glycine rich component in enamel has been given the word enamelin. It is therefore possible that what we are detecting are two classes of protein. It is also possible that with the growing evidence for the presence of proteases in the enamel that we may have to look at the possibility that they are playing a role in the not-expected glycine curve. Possibly the glycine is being incorporated by a group of proteins other than amelogenins. We were just surprised that the amount was so low because that wasn't what we had expected.

H. Slavkin: When you did the arithmetic to account statistically for your results, one impression I get is the notion that all of the

radioactivity that went into the enamel matrix, as we heard in the earlier presentations, is going back allegedly into the post-secretory epithelium, which function like osteoclast-like cells. Could you elaborate on whether the arithmetic works out that way and can you account for it?

C. Smith: It is in fact much more complicated because of the time constraints I had. We have kept to both short term intervals and to long term intervals. If the short term experiments are followed to long term, the curve continues to peak and build, and an artificial peak is maintained throughout the phase where the main bulk of amelogenins are being degraded. This suggests that we are recycling some of that radioactivity, which is a phenomena I believe is a little bit different than the initial event, which is more related to the bulk, initial secretory activity. But it does look like there is substantial reutilization in this system.