

DEGRADATION OF NEWLY FORMED ENAMEL PROTEINS IN RELATION TO THE SECRETORY ACTIVITY OF AMELOBLASTS

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INTRODUCTION

One mechanism postulated for the removal of enamel proteins (EPs) during the maturation of enamel is their active resorption by ameloblasts¹. The extensive lysosomal system of these cells has been cited as evidence supporting this route^{2,3}. This concept has also received support from immunocytochemical studies which have identified EPs in lysosomal elements of ameloblasts, particularly multivesicular bodies (mvbs)⁴. However, recent studies have raised the possibility that the EPs in lysosomes may not represent resorbed, aged proteins but rather that a portion of them derive from the post-translational degradation of newly formed secretory products^{5,6}. In the present paper we report our ongoing efforts to elucidate the origin of the EPs found within lysosomes using inhibitors of synthetic and degradative pathways.

MATERIALS AND METHODS

Biochemical analyses: enamel samples for SDS-PAGE and immunoblotting were obtained and processed as described previously^{5,7}. Briefly, sequential strips of early and late secretory stage enamel, and early and midmaturation stage enamel were dissected from the mandibular incisors of male Wistar rats (~100g). The proteins in these strips were extracted in a 1:1 mixture of PBS-Triton X-100 and sample preparation buffer, and then separated on 12% SDS-polyacrylamide gels. Some gels were stained with silver while the proteins from others were electrotransferred to nitrocellulose paper for immunoblotting with a rabbit antibody to mouse amelogenins (courtesy of H.C. Slavkin) followed by an alkaline phosphatase-conjugated secondary antibody.

Radioautographic studies: six male Wistar rats (~100g) were injected with ³H-methionine and sacrificed in pairs, at 10, 20 and 60 minutes following injection by perfusion fixation. The incisors were embedded in Epon and sections were processed for LM radioautography as described previously^{6,7}. Some samples were also prepared for EM radioautography.

Studies with inhibitors: two male Wistar rats (~100g) were injected intraperitoneally with 12 mg of leupeptin and an additional two with 10 mg of aprotinin over a period of 8 hours. Six other rats were injected with 3 mg/100g body weight of cycloheximide and sacrificed in pairs at 20, 60 and 120 minutes following injection. Treated rats, as well as uninjected controls, were perfused with glutaraldehyde and the incisors embedded in Epon⁴.

Analyses of lysosomes: the frequency of lysosomes in ruffle-ended ameloblasts from the initial 2 mm of the maturation stage was evaluated on random micrographs of the supranuclear compartment. Profile area measurements were made using a Zeiss MOP-3 manual image analyzer⁴.

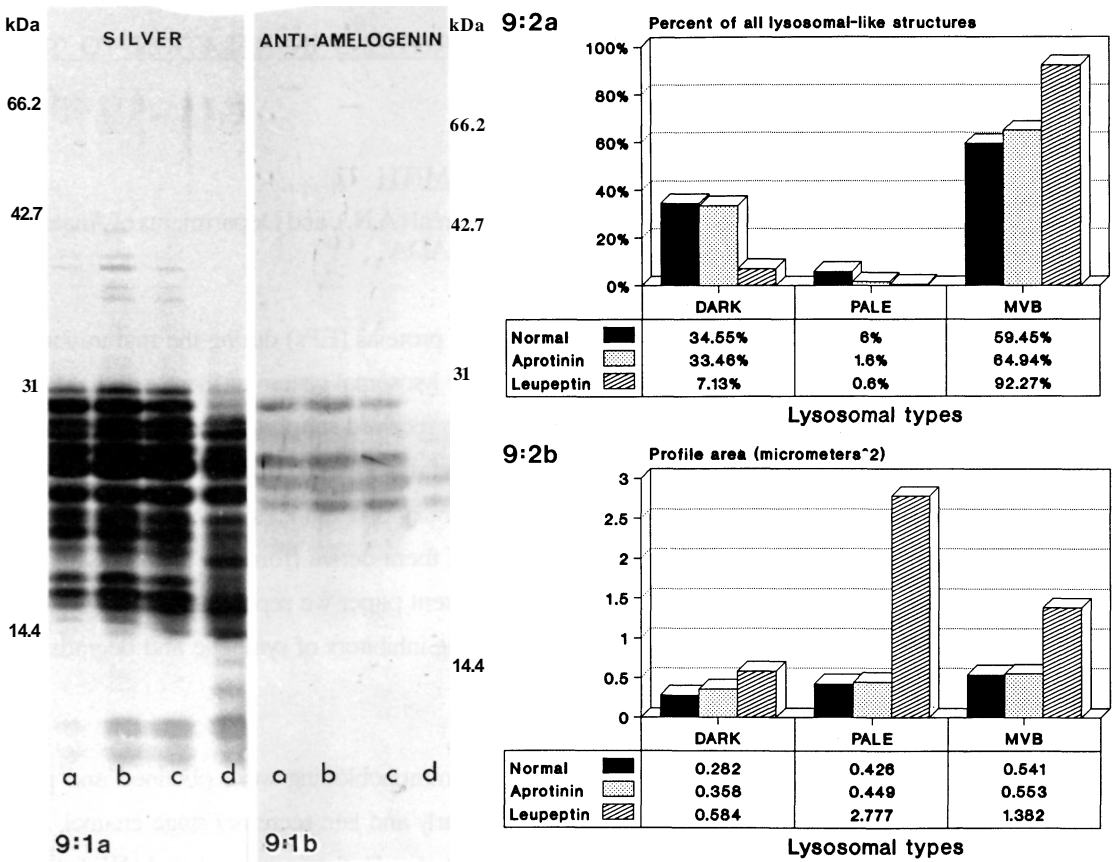


Figure 9:1. SDS-PAGE and immunoblotting of proteins extracted from early (a) and late (b) secretory stage, and early (c) and mid- (d) maturation stage rat incisor enamel. **1a:** 12% polyacrylamide gel stained with silver, **1b:** Immunoblot of a similar gel processed with anti-amelogenin IgGs followed by an alkaline phosphatase-conjugated secondary antibody.

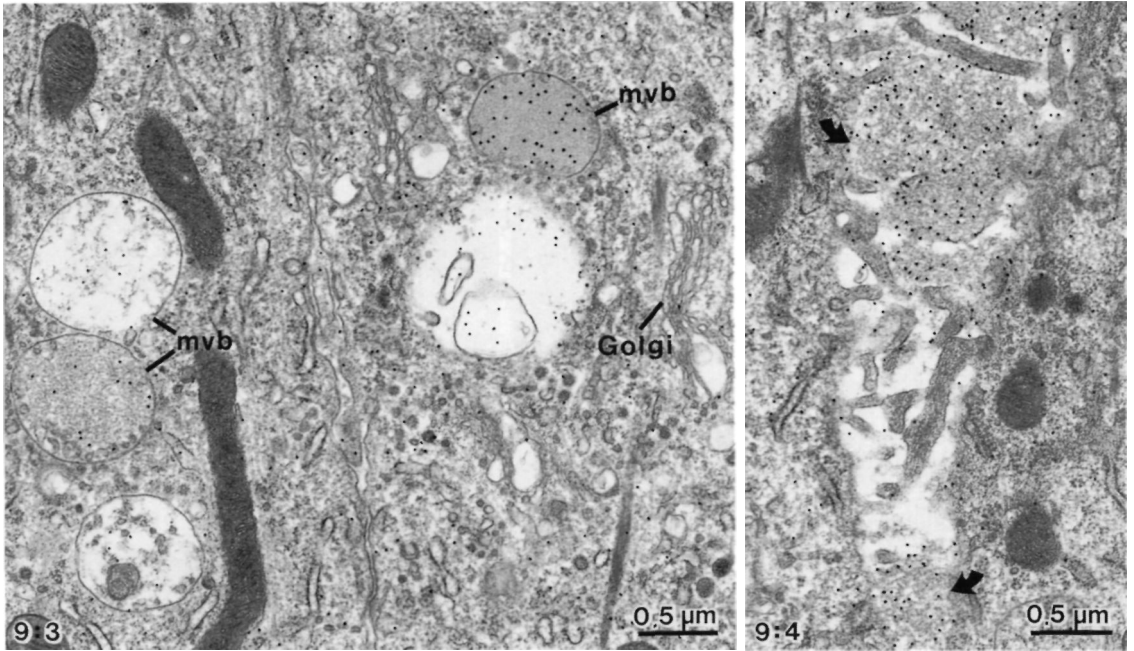
Figure 9:2. Bar graphs illustrating the relative proportion (a) and average profile area (b) of dark and pale lysosomes, and mvbs in normal, aprotinin- and leupeptin-treated ameloblasts from the initial 2 mm of the maturation stage.

Immunocytochemical labelling: tissue sections from treated and control rats were incubated with the anti-amelogenin antibody (diluted 1/50-100) revealed with protein A-gold as described previously⁴.

RESULTS

Silver staining of EPs proteins separated on 12% SDS-polyacrylamide gels showed several proteins between 14-32 kDa, as well as some around 6-10 kDa (fig. 9:1a). While many of the proteins diminished in staining intensity by the midmaturation stage, those near 6-10 kDa did not (fig. 9:1a). Only proteins between 14-32 kDa reacted with the antibody to amelogenins on immunoblots (fig. 9:1b).

Analysis of the relative frequency of lysosomes in ruffle-ended ameloblasts of control and inhibitor-treated rats indicated that in all cases the predominant lysosomal types were mvbs followed by dark lysosomes (fig. 9:2a). Aprotinin did not have any obvious effect on the relative frequency and profile area of the various lysosomes compared to control rats (figs. 9:2a, 2b, 3), but in some cases seemed to induce the accumulation of large amounts of immunolabeled material between maturation stage ameloblasts



Figures 9:3,4. Early maturation stage ruffle-ended ameloblasts from aprotinin-treated rats show ultrastructural features similar to normals, with the exception of frequent accumulations of immunolabeled material between cells (arrows). Golgi, Golgi apparatus.

(fig. 9:4). Leupeptin, however, caused a dramatic increase in the frequency of mvbs (fig. 9:2a). These mvbs were larger than the ones found in normal or aprotinin-treated rats (figs. 9:2b, 5) and were intensely immunolabeled (figs. 9:5, 6)). Typical leupeptin-induced dense bodies, which showed a variable immunolabeling, were also observed (fig. 9:6). Mvbs of normal rats showed some silver grains as early as 10-20 minutes following the injection of ^3H -methionine and were clearly radiolabeled by 1 hour (fig. 9:7).

Initial qualitative results from animals treated with cycloheximide showed few secretory granules in Tomes' processes and a variable but noticeable reduction in the relative number of certain subclasses of mvbs, particularly in presecretory stage ameloblasts.

DISCUSSION

Conceptually, only the lysosomes of ameloblasts in the maturation stage, where proteins are massively lost, should be immunolabeled if the EPs in them represent resorbed, aged material. However, EPs have been immunodetected in lysosomes of ameloblasts from the presecretory to the maturation stage^{4,5}, suggesting that either ameloblasts resorb EPs throughout amelogenesis or that a portion of the EPs in lysosomes derive from a route other than endocytosis. Indeed, EPs have been immunodetected in the lysosomes of ameloblasts very early during the presecretory stage, when few EPs are found extracellularly^{5,8} and ameloblasts presumably have had little time for resorption. Furthermore, ameloblasts at the beginning and end of the secretory stage, and during the maturation stage, do not transfer to the enamel all the radiolabel they incorporate following injection of ^3H -amino acids⁶.

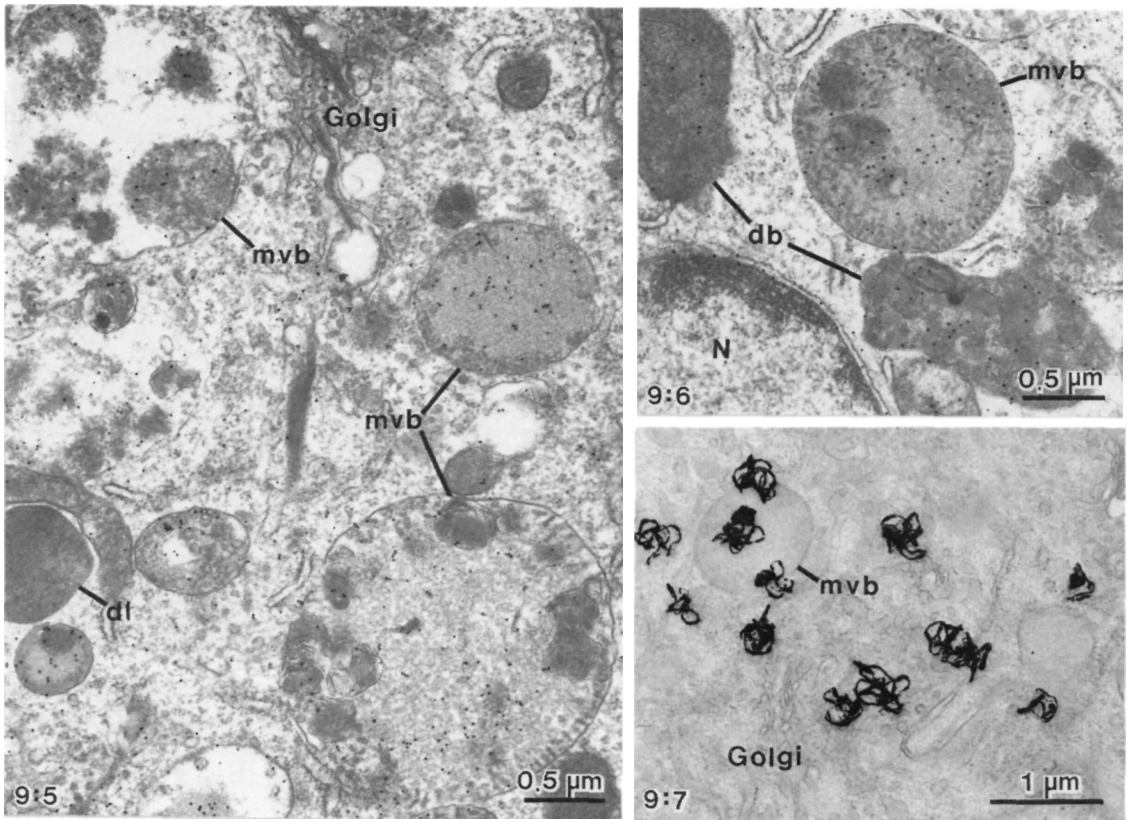


Figure 9:5.6. The mvbs in leupeptin-treated ameloblasts from the early maturation stage are more abundant and larger than those in normal or aprotinin-treated cells (compare with fig. 9:3). The density of immunolabeling over lysosomes, however, appears similar with both inhibitors. Numerous irregular dense bodies (db), variably immunolabeled, are also observed in leupeptin-treated ameloblasts (fig. 9:6). dl, dark lysosome; Golgi, Golgi apparatus; N, nucleus.

Figure 9:7. EM radioautography of early secretory stage ameloblasts following injection of ^3H -methionine in normal rats. At 10 minutes, silver grains are present over the Golgi apparatus (Golgi) and occasionally over mvbs.

In the rat incisor amelogenins are broken down extracellularly to low molecular weight peptides of 10 kDa and lower⁷. The antibody we use does not recognize, or has little affinity for, the smaller breakdown products of amelogenins. Although the possibility that the antibody cross-reacts with lysosomal enzymes cannot be excluded, the immunolabeling within lysosomes more likely represents intact or partially degraded amelogenins (above 14 kDa). Assuming that no low molecular weight degradation products are endocytosed, it could be postulated that these small peptides leave the enamel by a non-resorptive mechanism, perhaps by passively diffusing between cells.

Radioautographic studies with ^3H -methionine, an amino acid found in relatively high proportions in rat enamel¹⁰, have shown that leupeptin-treated ameloblasts secrete normal amounts of radioactive proteins but contain higher than normal counts intracellularly at 1 hour following injection⁶. This higher intracellular labeling has been suggested to represent the accumulation in lysosomes of newly synthesized proteins prevented from being degraded by the enzyme inhibitor⁶. The relative newness of proteins within

mvbs is further suggested by the finding that radiolabeling is seen in them as early as 10-20 minutes following injection of ^3H -methionine in normal rats. The present observation of an increase in frequency and size of mvbs containing immunolabeled material is consistent with the notion that EPs accumulate within mvbs of leupeptin-treated rats. The increase in frequency also implies that inhibition of thiol proteinases (serine proteinases are generally not found in lysosomes), or the resulting lack of degradation of EPs within mvbs, affects their turnover. Inhibition of the extracellular degradation of EPs by aprotinin in maturing enamel⁷ seems to have little effect on the frequency and size of lysosomes, a finding expected if the EPs in lysosomes do not mainly derive from aged, extracellularly processed proteins. The accumulation of immunolabeled material between ameloblasts may reflect the fact that proteins have not been sufficiently processed extracellularly to allow either their resorption or passive diffusion through the enamel organ. Such material, which is also occasionally observed between ameloblasts of normal rats⁴, may eventually be endocytosed via the lateral cell membrane. The finding of a reduction in relative number of certain subclasses of mvbs observed following inhibition of protein synthesis with cycloheximide does not allow us to conclude whether the loss of mvbs results from the lack of synthesis of EPs or lysosomal components. However, this result indicates that mvbs have a short half-life of probably less than 1 hour.

In summary, our studies favor the hypothesis that not all of the EPs in lysosomes represent resorbed, aged proteins but rather a portion of their contents are recently biosynthesized. Ameloblasts, as is the case for other cell types⁸, may, therefore, down-regulate their secretory activity by post-translational degradation of a portion of their products. (*Supported by the Medical Research Council of Canada*).

REFERENCES

1. Reith E.J., Cotty V.F. (1967) The absorptive activity of ameloblasts during the maturation of enamel *Anat. Rec.* 157: 577-588.
2. Katchburian E., Holt S.J. (1969) Role of lysosomes in amelogenesis. *Nature* 223: 1367-1368.
3. Ozawa H., Yamada M., Uchida T., Yamamoto T., Takano Y. (1983) Fine structural and cytochemical studies on the Golgi-SER system of ameloblasts with special reference to its resorptive function. In: *Mechanisms of tooth enamel formation*, Suga S., ed. Quintessence Publishing Company, Tokyo, pp 17-48.
4. Nanci A., Slavkin H.C., Smith C.E. (1987) Application of high-resolution immunocytochemistry to the study of the secretory, resorptive, and degradative functions of ameloblasts. *Adv. Dent Res.* 1: 148-161.
5. Nanci A., Ahluwalia J.P., Pompura J.R., Smith C.E. (1989) Biosynthesis and secretion of enamel proteins in the rat incisor. *Anat. Rec.* 224: 277-291.
6. Smith C.E., Nanci A. (1989) Secretory activity as a function of the development and maturation of ameloblasts. *Connect Tiss. Res.* 22: 147-156.
7. Smith C.E., Pompura J.R., Borenstein S., Fazel A., Nanci A. (1989) Degradation and loss of matrix proteins from developing enamel *Anat. Rec.* 224: 292-316.
8. Slavkin H.C., Bessem C., Bringas P., Zeichner-David M., Nanci A., Snead M.L. (1988) Sequential expression and differential function of multiple enamel proteins during fetal, neonatal, and early postnatal stages of mouse molar organogenesis. *Differentiation* 37: 26-39.
9. Bienkowsky R.S. (1983) Intracellular degradation of newly synthesized secretory proteins. *Biochem. J.* 214 1-10.
10. Robinson C., Lowe N.R., Weatherell J.A. (1977) Changes in amino acid composition of developing rat incisor enamel *Calif. Tissue Res.* 23: 19-31.

Poster 9

Deutsch: The degradation of enamel protein in the early stages of formation, has been shown not only morphologically by identifying lysosomes, but by analytical biochemistry, Dr. Nanci, you say there is a substantial proportion of lysosomal activity shunting the protein. What proportion are you speaking about?

Nanci There is a certain amount of degradation of enamel proteins which occurs extracellularly during the secretory stage of amelogenesis. Smith and Nanci (Connect. Tiss. Res. 22:147-156) presented radioautographic data on the secretory activity of the ameloblasts showing that as much as 10% of the secretory output of these cells could be shunted into lysosomes.

Robinson: Is the antibody you use for identifying proteins in the cell, a monoclonal?

Nanci: The antibody we use is a polyclonal antibody (IgG fraction) raised in rabbit against mouse amelogenin (courtesy of Dr. H.C.Slavkin). In the rat incisor it recognises primarily proteins near 14-32 kDa (Nanci et al., Anat. Rec. 224-291, 1989).

Robinson: There are some of the proteases in the enamel matrix in this molecular weight range and I am concerned that you may be partly identifying proteinases in the lysosomes.

Nanci: Dr. Smith in poster 35, shows proteolytic activity in this molecular weight range. So we cannot be sure whether the antibody also recognises extracellular proteinases. The multivesicular bodies of ameloblasts at all stages of amelogenesis are labelled, but not those of other cells of the enamel organ, incisor odontoblasts related to secretory and maturation stage enamel, dental sac pulp and kidney cells. Even though recognition of an ameloblast lysosomal enzyme cannot be totally ruled out, we feel that the immuno-labelling in lysosomes represents enamel proteins. The retention of radio-label in ameloblasts, as well as the increase in the size and frequency of multivesicular bodies following leupeptin treatment, (a drug which inhibits the cathepsins in lysosomes), suggests the shunting of enamel proteins in lysosomes.

Snead: The question of antibody specificity was recently revisited in our laboratory. To remove the possibilities of contaminating antigen obtained from purified enamel we have made antipeptide antibodies by artificially synthesizing amino acid peptide sequences contained within the intact mouse amelogenins. This will ensure that the antigen is not contaminated by anything other than what was synthesized by the machine. Each purified synthetic peptide is used to immunize rabbits and the polyclonal antibody, having the specificities of a monoclonal antibody, have been used in a number of immuno detection techniques. The antipeptide antibodies, are able to discriminate between a variety of isoforms of amelogenin, which have been discriminated by both size and charge. We are collaborating with Dr.Nanci and Dr. Smith to utilize anti- peptide antibodies to attempt to identify processional processing of amelogenins during biomineralisation.

Nanci: I have no comments on Dr. Snead's views, I would however like to add the piece of information that immunoblots of enamel organ proteins with anti-amelogenin antibody, stain uniquely in the molecular weight range of amelogenins. Furthermore, as Dr.Smith (poster 35) indicates, that much of the enamel organ-associated proteolytic activity resides above 30 kDa. The antibody shows little or no staining in this region.

McKee: Have you been able to determine, and I assume that this would be by electron microscopic autoradiography, what proportion of enamel proteins might leave the enamel by an extracellular route between cells compared to an intracellular degradation route?

Nanci: The enamel proteins in lysosomes, could be derived either from resorptive activity, or from the shunting of newly synthesized proteins from the secretory organelles into lysosomal elements. However, at this moment we cannot dissociate the two routes. If one assumes that the lysosomes all originate from post-translational degradation, then where does the protein which is degraded extracellularly go? This is an issue to which we have no clear answer yet.

Deutsch: Does your antipeptide antibody now recognise the small protein 14 kDa and below?

Snead: Yes

Robinson: Can you say categorically that this antibody does not recognise proteinases? From a number of other fields antibody work with proteinase is very difficult, and they often react with antibodies from very unrelated sources.

Nanci: We cannot rule out the possibility that the antibody recognises proteinases, particularly extracellular ones. However enzymes are usually not very resistant to strong fixation conditions and embedding in Epon. With the anti-amelogenin antibody we see immunolabelling in lysosomes under relatively harsh tissue processing conditions in ameloblasts, but not in various other cell types.

Snead: I think it is very difficult when using a polyacrylamide gel electrophoresis (PAGE) or high pressure lipid chromatography (HPLC) purified amelogenin protein as an immunogen to say with a great deal of finality, precisely what contaminants are co-purified. If you were asking me would I bet my life, that it does not possibly contain antibodies directed against proteins other than amelogenins, I would have to say, "I would rather not take that bet". However I believe our lab. has demonstrated that the antibody is specific for amelogenins.

Young: I am a little concerned about the interpretative jump that has been made with the idea that this is a recycling of excess protein. I wonder if any thought has been given to the possibility that in enamel secretion, one is dealing with a pre-amelogenin or a pre-enamelin type of protein which then requires some form of proteinase to activate it in the gel outside the cell? Consequently as in the parallel with the pro-collagen peptidases, the lysosomal activity in the secretory cells may be somewhat of a processing step prior to the formation of the extracellular gel.

Nanci: The reason why we have used the term excess, is that the beginning and end of the secretory stage, and during the maturation stage, ameloblasts incorporate more radiolabeled amino acids than they secrete into the enamel. This does not happen during the mid-portion of the secretory stage.

Limeback: First, a comment to Dr.Young, connective tissue collagen fibres are formed following pro-collagen secretion and processing by the pro-collagen peptidases which are not associated with lysozymes. I also have a question for Dr. Nanci. Your Western blots show a number of amelogenin bands that are stained by your antibody. What is the smallest protein that reacts with this antibody?

Nanci: The smallest proteins resolvable on 12 SDS-polyacrylamide gels stained with silver, range near 6-10 kDa. On Western blots, the smallest protein ranges near 18 kDa.

Limeback: If the rat amelogenins are analogous to bovine and porcine amelogenins, then most of them are partially processed intermediate peptides that have a common N-terminal amino acid sequence. Since your antibody does not stain proteins smaller than 18 kDa, it must be recognising an amino acid sequence of the molecule somewhere in the middle.

Nanci: That is a very good possibility.

Snead: At a preview "Enamel" meeting, my colleague Dr.Slavkin suggested a pre-pro-amelogenin, with cleavage of the leader sequence used to direct the egress of the secretory protein. This was later confirmed by Dr. Sasaki and Dr. Shimokawa with cDNA data from the bovine model, in which a leader sequence was identified. The antipeptide antibodies were designed to attempt to answer questions about the processing of amelogenins, in order to examine whether or not various domains of the amelogenins are differentially retained within or upon enamel hydroxyapatite surfaces, or in the extracellular milieu, which many people believe is responsible for organising the crystallites of enamel. It is not yet known whether or not processing occurs, processionaly from the carboxyl or amino terminus of the amelogenin protein during mineral maturation. We have synthesised various epitopic domains unique to the amelogenin which span the intact molecule, but we are not sure if the first cleavage occurs at the tyrosin-rich polypeptide (TRAP)-end or the carboxyl end of the molecule. Recently, we described a carboxyl-terminal peptide extension containing hydrophilic amino acids which is never observed within the extracellular matrix. Perhaps, sequences at either end of the intact protein can participate during stoichiometric organisation of enamel proteins in the extracellular matrix.

Sasaki,S.: As a biochemist, I would like to make some comments on Dr.Nanci's study. Intracellular protein degradation at an early stage of protein synthesis, perhaps for the purpose of disposal of over production or misproduction of the protein, is well known, not only in collagen but also in other kind of proteins. I think it is very difficult to distinguish this type of nascent protein degradation, from degradation of amelogenin which is once secreted and then re-absorbed by ameloblasts. We found that the C-terminal peptide of amelogenin was cleaved at an early stage of enamel formation but we have not identified this peptide in the enamel matrix yet. That means this C-terminal peptide consisting of 12 amino acids must be absorbed and digested intracellularly, perhaps by lysosomes. I think the pulse-chase experiment that you have done is an excellent method to distinguish two different phenomena in the ameloblasts. In addition, nascent protein degradation may occur in the area close to the endoplasmic reticulum or the Golgi apparatus, and degradation of amelogenin components once secreted may happen in lysosomes which reside close to the enamel surface. I hope electron microscopists can differentiate morphologically the two separate phenomena.

Nanci: The comment is well taken and indeed it was one of our preoccupations. This is the reason we have performed radiolabelling experiments using ^3H -methionine at short time intervals. The other attempt we have made is with the drug leupeptin, ameloblasts secrete normal amounts of radio labelled products yet have higher than normal intracellular radioactivity (1 hour after the injection of ^3H -methionine). When leupeptin treated ameloblasts are examined morphologically, multivesicular bodies in particular, are not only more numerous but bigger as well. Since leupeptin inhibits cathepsin B, H, L in

lysosomes, it may be postulated that newly formed enamel proteins are prevented from breakdown and accumulate in lysosomes under the effect of this drug.

Aoba: Our results obtained by using nuclear magnetic resonance (H^1NMR) suggested that some regions of 25 kDa and 20 kDa amelogenin seems to be exposed on the molecular surface. Also, the C-terminal segment of the dislocated 20 kDa amelogenin keeps its flexibility of motion in solution.

Takuma: In response to Professor Sasaki's idea. In my poster No.31, I present some pictures of a very early stage of amelogenesis. As you see they have been taken from the differentiating ameloblasts and shows that enamel matrix is secreted before the establishment of the cellular polarity of the ameloblasts. This matrix reacts to the amelogenin antibody beautifully, and so it is undoubtedly a part of the enamel matrix. Since the cellular polarity is not yet established, the matrix is secreted in several directions not towards the dentine side, even in between the cells of the stratum intermedium. This type of matrix does not mineralise at all, so it should be absorbed in some way before the commencement of real amelogenesis. That might be related to Prof. Sasaki's idea I think.