Table I. A comparison of the stability of the 12,000 × *g* supernatant and plasmin-insoluble percipitate to 4 enzymes

Enzyme	A) 12,000 × *g* supernatant		B) Plasmin-insoluble precipitate		C) Plasmin-insoluble precipitate	
	Mortality	Day of 50% mortality	Mortality	Day of 50% mortality	Mortality	Day of 50% mortality
Incubation control (no enzyme)	7/10	22	9/10	20	10/10	15
DNase	8/10	19	9/10	18	9/10	20
RNase	7/10	20	3/10	—	10/10	20
Trypsin	10/10	19	2/10	—	2/10	—
Lysozyme	10/10	20	9/10	17	9/10	23

A 12,000 × *g* supernatant (A) and 2 separate preparations of plasmin-insoluble precipitate (B and C) were incubated with 4 enzymes. One ml aliquots were incubated at 37 °C with 0.1 mg of enzyme. Incubation time was 60 min for A and B and 75 min for C. Each incubation aliquot was equally divided among 10 mice.

freezing, incubation at 60°C for 3 min, and filtration through bacterial retaining filters. These characteristics cast some doubt on the non-cellular nature of 12,000 × *g* supernatant. Since FRUTH has demonstrated that one leukemic cell can probably transmit leukemia in the mouse, the criteria for any non-cellular preparation must be rigorus.

Although the 12,000 × *g* supernatant did not satisfy non-cellular criteria, the non-cellular transmission of the L-4946 leukemia in the CF1 mouse is suggested by stability of the subcellular extract in distilled water and the presence of protein and RNA but no DNA in the subcellular extract.

There is a significant difference between the centrifugation studies reported in this article and those reported by RENNERT[5]. In the CF1 mouse, leukemogenic activity could still be detected in a 125,000 × *g* supernatant as well as in the 30,000 × *g*, 60,000 × *g*, and 90,000 × *g* pellets. In the Swiss albino mouse, RENNERT found no leukemogenic activity in a 133,000 × *g* supernatant and little leukomegenic activity in the 30,000 × *g* and 133,000 × *g* pellets.

The observed difference could be explained by a difference in the minimal number of viral particles required to cause infectivity in the CF1 and Swiss albino mice. Centrifugal force of greater than 100,000 × *g* would certainly sediment the vast majority of viruses of the size RENNERT has described. Even though the percent viri remaining in these high-speed supernatants would be very small, the number of viri in a supernatant could still be large enough to infect a susceptible mouse strain, but not another, less susceptible strain. Extracted RNA from L-4946 ascites cells, the 12,000 × *g* supernatant or the plasma-insoluble precipitate were never leukemogenic in the CF1 mouse[3, 6–8].

Zusammenfassung. Mittels Ultrazentrifugation von infektiöser Ascitesflüssigkeit bei 125.000 × *g* und einem gereinigten zellfreien Extrakt konnte ein RNS-Virus als infektiöses Agens und Ursache der Leukämie und eines Lymphosarkoms nachgewiesen werden. Einzelne Mäusestämme erwiesen sich als resistent.

G. B. HUMPHREY[9, 10]

Table II. The protein and nucleic acid content of the 12,000 × *g* supernatant and plasmin-insoluble precipitate

Preparation	Mortality	Day of 50% mortality	Quantity per mouse	
			Protein (μg)	RNA (μg)
12,000 × *g* supernatant	8/10	22	3,200	–
Plasmin-insoluble precipitate	8/10	22	40	2.5

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The Decrease in the Concentration of Organic Material in the Course of Formation of the Enamel Matrix

Enamel maturation consists in the transformation of the previously secreted young enamel, rich in organic material and water and poor in calcium salts, into the highly calcified mature enamel. Briefly, there is a removal of organic material and water and addition of new calcium salts which crystalize in apatites. The loss of organic material has been shown by radioautographic[1–6] and biochemical studies[7–10]. Once the young matrix is

formed the secretory ameloblasts reduce themselves in height and these shortened ameloblasts are believed to be the agents of removal of both organic matrix and water[11–13].

Continuously growing teeth, that is, the incisors of rats and mice, were mostly used in such studies, since all stages of amelogenesis can be visualized or collected in a single tooth. Molar teeth from guinea-pigs, also continuously growing, were used in the present study. By correlating the rate of migration of ameloblasts after labeling with ^{3}H-thymidine, and the radioactive reactions over ameloblasts and enamel matrix after injection of ^{3}H-proline, it was hoped to establish where the loss of organic matrix took place.

Material and methods. Guinea-pigs, 2 days old, were injected i.p. with a single dose of either ^{3}H-thymidine (1 μCi/g of body wt.) or ^{3}H-proline (2.5 μCi/g of body wt.) and sacrificed at several time intervals after the injection. Molar teeth were removed, fixed in Bouin for 24 h, decalcified in 5% trichloroacetic acid and embedded in paraffin. 5 μm thick sections were processed for radioautography by the 'stripping-film' technique[14] using AR 10 plates from Eastman Kodak and post-stained with Giemsa at pH 5.6.

In a longitudinal mesio-distal section of the molar, several areas of odontogenesis can be seen. All the observations were made at the mesial or distal side of the tooth, dividing it into 6 zones, a slightly modified version of the classification used by WASSERMANN[11] (Figure 1). The length of each zone was determined in 60 enamel organs by counting nuclei of overlaid ameloblasts in a longitudinal row from the apical to the oral surface (Figure 1).

Results and discussion. Radioautographs from animals that received tritiated thymidine were examined and the position of the labeled ameloblasts, situated most distantly from the apical limit of the inner epithelium, was determined at each time interval after injection. 1 h after the injection of thymidine only ameloblasts in zone A were labelled, thus indicating their proliferative ability, which was maximal at position 119. At later time intervals, labelled nuclei from daugther cells appeared at more occlusal positions approaching the oral epithelium. By plotting the number of cell positions traveled by the uppermost labelled cell at each time interval (considering the position 119 as the starting point; Table I) and by calculating the linear regression, the rate of migration was determined as being 2.36 cells per h, i.e., each ameloblast took about 25 min to move from its position to the next immediately occlusal one.

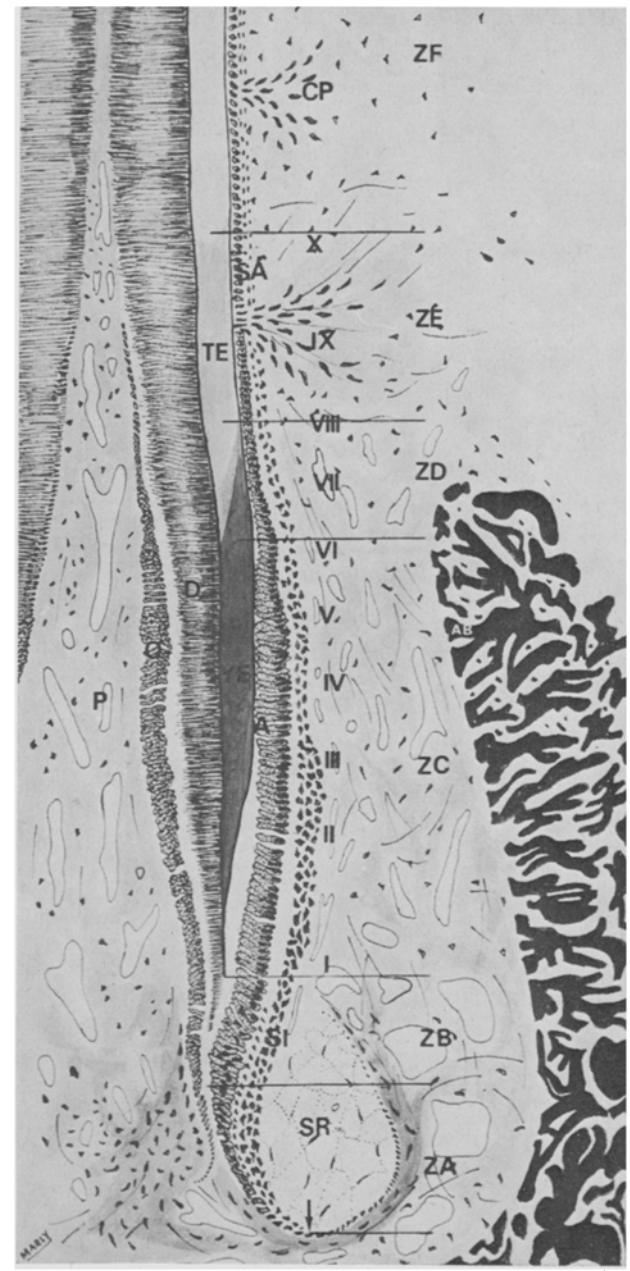

Fig. 1. Drawing showing part of the distal side of the guinea-pig molar and its division in zones and regions. Zone A (ZA) or embryonic zone, where the ameloblasts have mitotic ability, has an average of 123±2.5 overlaid ameloblasts; Zone B (ZB), where the ameloblasts (A) start their differentiation into secretory cells, has 51±1.7 cells; in Zone C (ZC), or secretory zone, the young enamel (YE) is formed and there are about 305±5.9 ameloblasts; in Zone D (ZD), the young enamel is gradually transformed into transitional enamel (TE) and the secretory ameloblasts change themselves into short ameloblasts (SA) which form a column of about 48±1.9 cells; Zone E (ZE) contains only transitional enamel and has 166±5.1 cells; Zone F (ZF) corresponds to the fully calcified mature enamel which dissolves after decalcification and has 337±13.8 cells. The full length of the distal (or mesial) side of the tooth has an average of 1,030±15 overlaid ameloblasts. Starting in zone D and increasing in number towards the oral epithelium, the column of ameloblasts is interrupted by periodontal fibers which form the cement pearls (CP). Zones C, D and E were subdivided in regions I to X corresponding to the cell positions given in Tables II and III. P, pulp; SI, stratum intermedium; SR, stellate reticulum; the arrow indicates the limit between the outer and the inner epithelium of the enamel organ.

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Silver grain counts over ameloblasts and enamel matrix, in the teeth of animal which received tritiated proline, were made in well determined regions of zones C, D and E, each region formed by an average of 15 overlaid ameloblasts. Grain counts over cells indicated that the most actively secretory ameloblasts were those of regions II to V (Table II). Secretory activity fails short in the upper part of the secretory zone (region VI), coinciding with the appearance of smaller ameloblasts.

In the enamel matrix the radioactivie reaction after a single injection of labelled amino acid displays a characteristic pattern which differs from other hard tissues. The first labelled material reached the matrix 30 min after the injection of tritiated proline and up to 1 h the reactions appeared as a well defined band close to the apical surface of ameloblasts of zone C. From 2 h on, this reactive band started to diffuse into the preformed matrix reaching its full thickness from 2 to 24 h later, depending on the region of the secretory zone (Figures 2 to 8). Radioactive material first appeared in the matrix of zones D and E, 2 and 24 h after the injection, respectively.

Assuming that enamel matrix moves up at the same rate as the ameloblasts and by correlating the results on Table III, we found that with time there was a reduction in the concentration of labelled material. For instance, the cells and matrix which at 24 h after the injection were in region II will be in region III 24 h later, i.e., 48 h after the injection of labelled proline, and the silver grain concentration in the latter is less than half the former. The same occurred in the other regions as can be seen in Figure 8; after the peak of reaction was reached, a decline was observed when the structures were still in the secretory zone; and the more occlusal the region the smaller was the peak and the decline of reaction. This could be explained either as a dilution by the unlabelled material coming in after all the labelled precursor was no longer available, or as a removal of part of the labelled material.

Table I. Cell position of the labelled ameloblast situated most distantly from the apical limit of the inner epithelium of the guinea-pig molar and the number of positions migrated at various time intervals after a single injection of ^{3}H-thymidine.

Time (h)	Cell position[a]	No. of positions migrated
1	119	0
12	126	7
24	171	52
48	228	108
96	347	228
144	455	336
240	582	463
360	768	648

[a] Mean of 2 teeth; the cell position was normalized according to the total number of a column of ameloblasts and the number of ameloblasts of each zone (Figure 1).

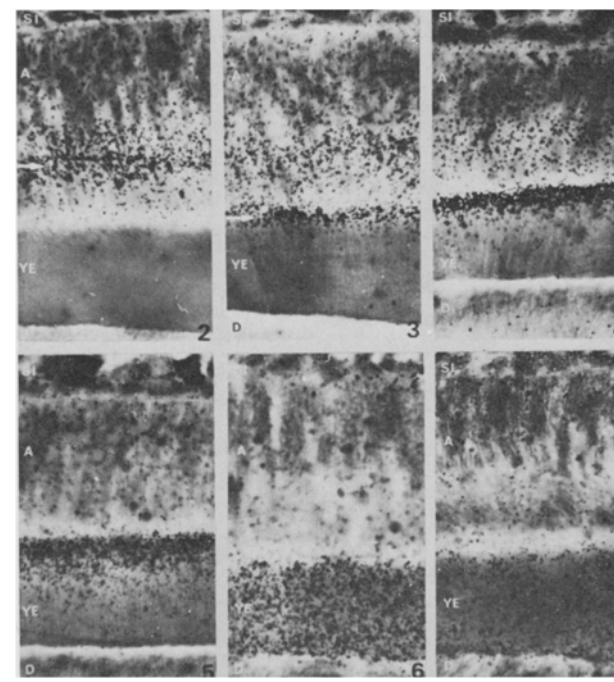

Fig. 2 to 7. Radioautographs of the middle region of zone C (regions III or IV, Figure 1) from guinea-pig molars at various time intervals after the injection of ^{3}H-proline. Giemsa, × 330. SI, stratum intermedium; A, ameloblasts; YE, young enamel; D, dentin. 10 min after the injection (Figure 2) a strong reaction was present at the supranuclear region (arrow) of ameloblasts; 30 min after the injection (Figure 3) another band of reaction appeared over the region of Tomes' processes and adjacent young enamel matrix (pre-enamel); 1 h later (Figure 4) the reaction over ameloblasts was declining while the band over the adjacent matrix became more concentrated in silver grains; at 6 h (Figure 5) this reactive band was stronger and scattered silver grains started to diffuse through the unlabeled matrix, reaching its full thickness 24 h after injection (Figure 6); at that time the matrix became uniformly labeled and the silver grains attained maximum concentration; at 48 h (Figure 7) this concentration was lower indicating a removal of labelled material.

Table II. Concentration of silver grains over ameloblasts in different regions of zones C, D and E of guinea-pig molar at various time intervals after a single injection of ^{3}H-proline

Time (min)	Zones / Regions	C I	II	III	IV	V	VI	D VII	D–E VIII	E IX	X
	Cell Positions	175–188	268–281	318–331	369–382	420–433	466–479	496–509	520–533	603–616	680–693
10		11.3 (1.0)	17.8 (1.2)	16.9 (1.3)	17.5 (1.5)	18.4 (1.4)	5.5 (0.8)	2.9 (0.5)	2.8 (0.6)	1.0 (0.4)	0.6 (0.2)
30		12.7 (0.9)	23.9 (1.0)	23.8 (1.1)	24.2 (1.6)	21.7 (1.4)	9.9 (1.0)	7.7 (0.6)	6.8 (0.8)	4.9 (0.6)	4.6 (0.6)
60		9.3 (0.7)	15.1 (0.7)	14.7 (0.6)	17.5 (0.8)	13.0 (1.0)	8.4 (0.9)	6.1 (0.7)	5.7 (0.7)	6.1 (0.5)	6.6 (0.6)

Each figure represents the mean number of silver grains per 42 μm^2 counted in 40 of such areas over 14 to 16 cells per region of 2 teeth. The standard error is given in parentheses.

Table III. Concentration of silver grains over enamel matrix of zones C, D and E of the guinea-pig molar at various time intervals after a single injection of ^{3}H-proline

Time	Zones	C						D	D–E	E	
	Regions	I	II	III	IV	V	VI	VII	VIII	IX	X
	Cell Positions	175–188	268–281	318–331	369–382	420–433	466–479	496–509	520–533	603–616	680–693
10 min		0	0	0	0	0	0	0	0	0	0
30 min		30.3 (1.5)	18.3 (3.1)	11.7 (2.3)	6.3 (1.2)	2.8 (0.6)	0.8 (0.2)	0	0	0	0
1 h		52.2 (3.1)	26.5 (3.1)	14.8 (2.2)	8.7 (1.4)	4.2 (0.8)	0.4 (0.1)	0	0	0	0
2 h		44.8 (3.0)	32.1 (1.8)	22.9 (2.2)	15.5 (1.6)	10.0 (1.2)	2.0 (0.3)	0.4 (0.1)	0	0	0
6 h		46.2 (2.1)	38.2 (1.4)	30.7 (1.8)	19.6 (1.5)	9.9 (1.0)	3.4 (0.5)	0.3 (0.8)	0	0	0
24 h		22.9 (1.4)	36.4 (1.2)	35.2 (0.8)	31.7 (0.5)	27.3 (0.6)	15.3 (0.9)	4.2 (0.3)	0.9 (0.1)	0.3 (0.0)	0
48 h		7.8 (0.7)	12.9 (0.5)	14.3 (0.4)	13.9 (0.4)	13.4 (0.4)	11.2 (0.5)	7.0 (0.4)	4.6 (0.3)	0.1 (0.0)	0
96 h		2.2 (0.4)	3.2 (0.3)	3.4 (0.3)	3.4 (0.3)	5.8 (0.3)	10.4 (0.3)	13.9 (0.4)	13.6 (0.3)	15.8 (0.4)	0.3 (0.0)
240 h		0.3 (0.2)	0.2 (0.1)	0.1 (0.4)	0.1 (0.0)	0.3 (0.0)	0.8 (0.1)	1.2 (0.1)	1.3 (0.1)	2.1 (0.1)	0.9 (0.1)

Each figure represents the mean number of silver grains per 42 μm^2. The silver grains were counted in the whole thickness of the enamel matrix of each region of 2 teeth (the same as in Table II) divided by eye piece reticulum in as many as possible squares of 42 μm^2 each. The standard error is given in parentheses.

Biochemically, DEAKINS[15] had demonstrated that in the process of enamel calcification 'the influx of calcium salts is compensated for chiefly by loss of water and, to a lesser extent, by loss of organic material'. Such loss of organic material is also referred to by other authours[7–10, 18] dealing with the subject.

Recently it has been described that secretory ameloblasts have a high content of lysosomes which are imputed as responsible of extracellular degradation of organic matrices[16, 17].

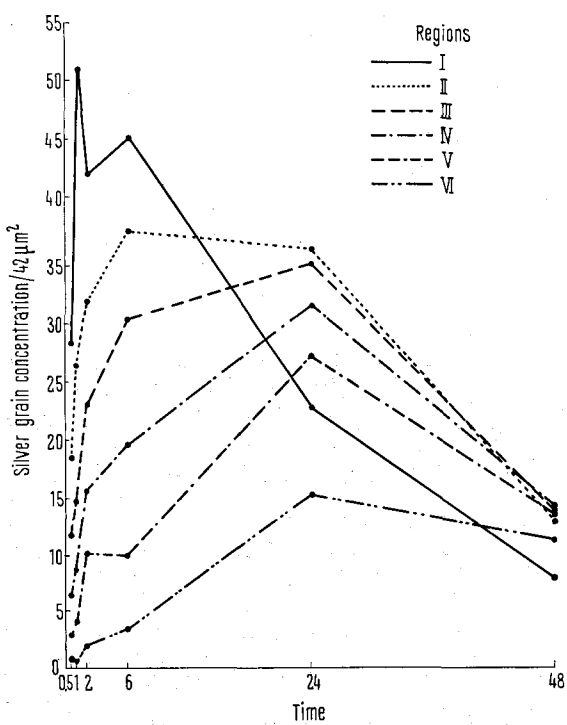

Fig. 8. Silver grain concentration over regions of zone C from the guinea-pig molar at various time intervals after injection of ^{3}H-proline. The intensity of reaction declined from the apical towards the occlusal end of zone C. In regions III to VI, where the young enamel matrix attains its full thickness, the peak of silver grain concentration occurred 24 h after the injection. In regions I and II this peak was reached at earlier time intervals. In all regions, except region VI, a lower silver grain concentration was found when the matrix was still in the secretory zone.

On the other hand, once the cells and matrix went through zones D and E (regions VII to X) the matrix appeared relatively stable as far as protein material was concerned (the same was found when other labeled amino acids and sulfur were used; BLUMEN and MERZEL, in press). Indeed, by comparing the data in the same Table III, the silver grain concentrations of matrix in region VI at 24 h and region IX at 96 h (72 h being the time for cells and matrix to move from one to the other) were almost identical; the same could be observed by comparing regions V and VIII at 48 h and 96 h. These results suggest that the organic material of enamel matrix was labile in the secretory zone and relatively stable in post-secretory zones. DEAKINS[15] also stated that, chronologically, the organic material is lost first and water later, and that the increase in hardness occured only in the period during which water was lost while the amount of organic matrix remains constant.

Thus our results suggest that one of the steps of enamel maturation, the removal of organic material, is related to the activity of the secretory ameloblasts.

Resumen. La formación de la matriz orgánica del esmalte fué estudiada, a través de la radioautografia, en molares de cobayas que recibieron Timidina-^{3}H y Prolina-^{3}H. Los resultados parecen indicar que la remoción de material orgánico de la matriz, una de las etapas del proceso de maduración del esmalte, están en la dependencia de los ameloblastos secretores y no de los ameloblastos reduzidos como ha sido propuesto.

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