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# REVERSIBLE AND IRREVERSIBLE EFFECTS OF TEMPERATURE ON AMELOGENESIS OF HAMSTER TOOTH GERMS *IN VITRO*

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## Abstract

Hamster first hamster molar tooth germs in early secretory stage of amelogenesis were cultured for one day *in vitro* at 6°C, 22°C, 37°C or 45°C in the presence of $^{3}$H-proline, $^{45}$Ca and $^{32}$P-orthophosphate. Other explants were cultured without these labels and after culture examined by histology. The highest temperature tested was lethal to the explants, decreased total dry weight and rapidly increased total uptake of the radiolabelled mineral ions, probably merely due to physicochemical modification of the existing preculture minerals. Optimal synthesis and secretion of amelogenins were measured at physiological temperature (37°C). Effects of exposure to both temperatures below the physiological value were virtually reversible when explants were grown at physiological temperature (37°C) for another day. However, amelogenin secretion during this recovery period did not reach values as high as those found for the first day in explants initially grown at physiological temperature during the first day.

We concluded from the four temperatures examined that the optimal temperature for enamel matrix deposition *in vitro* was 37°C. At this temperature enamel biosynthesis and its secretion are high. Lowering the temperature slows down the metabolism without any apparent harmful effect. Normal development of the tooth explants *in vitro* resumes when the culture temperature is restored to physiological levels (37°C). For temporary storage of tooth germ explants prior to any reimplantation, we therefore recommend a temperature of 6°C.

**Key Words**: Enamel matrix, mineralization, temperature, tooth organ culture, preservation of tooth germs.

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## Introduction

In view of the possibility to store temporarily healthy tooth germs outside the body prior to their transplantation or reimplantation it is important to know what the effect is of the temperature for maintaining explants viable in a culture medium *in vitro*. But up to now, in only one *in vivo* study Kreshover and Clough (1953) examined effects of elevated temperature on tooth formation in pregnant rats as well as in their offsprings by keeping the pregnant mothers in an incubator at increased temperature (simulating fever). They reported changes in amelogenesis ranging from ameloblastic dysfunction to complete cellular degeneration with arrest of enamel matrix formation, both in the mothers and their offspring. Dentinogenesis, however, was affected only in the rat pups but not in the mothers. However, up to now, it is still unknown how temperature influences tooth formation over a more extended temperature range and to what extent such effects are reversible mainly because of lack of a suitable *in vitro* organ culture system.

After improvement of an organ culture system upto the level that we could obtain normal enamel formation *in vitro* (Bronckers *et al.*, 1983), it became possible to study the effects of refrigerator temperature (6°C), room temperature (22°C), physiological temperature (37°C), and high temperature (45°C) mainly on enamel matrix synthesis, secretion and enamel mineralization in a 24-hour organ culture. Furthermore, we investigated whether the temperature-induced changes, if any, were reversible by allowing the explants to recover for another 24 hours *in vitro* under standard conditions at 37°C.

## Materials and Methods

### Culture procedures

In three analogous experiments, pairs of first maxillary molars from 3-day old hamsters were excised aseptically and put on top of Millipore filters placed on metal grids and kept in holding medium (see below) until all dissection procedures were completed. Then, three experimental explants were transferred to fresh culture

medium adjusted to each one of the temperatures under investigation (6°C, 22°C, 37°C or 45°C).

In the first set of experiments, $^{3}$H-proline, $^{45}$Ca and $^{32}PO_4$ (5 $\mu$Ci/ml each) were added to the medium during the first day of culture; after 24 hours the experiments were terminated and explants analyzed.

In a second series of experiments, three tooth explants were initially cultured for 24 hours at each of the four temperatures in culture medium without any radiolabels. On the second day, the cultivated teeth were transferred to fresh culture medium to which the radiolabels were added; all explants were subsequently grown for 24 hours at 37°C.

The specimens intended for histology were cultured without radiolabels. Holding and culture media consisted of $BGJ_b$ medium, supplemented with 15% fetal bovine serum, 250 $\mu$g/ml freshly dissolved vitamin C, 200 $\mu$g/ml glutamine (Merck, Darmstad, FRG), 50 $\mu$g/ml penicillin-G and 30 $\mu$g/ml streptomycin sulphate (Sigma Chemicals, St.Louis, USA). The culture dishes were incubated in humidified jars under an atmosphere of 50% $O_2$, 5% $CO_2$ and 45% $N_2$ at 37°C (Bronckers *et al.*, 1983).

### Histological procedures

After culture (second set of experiments) the explants were fixed for 1 hour in freshly prepared 3% glutaraldehyde, containing 0.01% $OsO_4$ in 0.1 M sodium cacodylate (pH 7.3) in melting ice. Some explants were postfixed in 1% $OsO_4$ (pH 7.4) for 1 hour at 4°C in the same buffer. Embedding and staining procedures were as reported by Bronckers *et al.* (1983). Semi-thin sections (0.5-1 $\mu$m) were cut with glass knives and stained in 1% toluidine blue in 1% sodium tetraborate.

### Chemical extraction procedures

After radiolabelling, the explants were briefly rinsed in unlabelled culture medium to remove any adhering radioactivity and subsequently freeze-dried. After weighing, explants were sequentially extracted using a three step extraction procedure (Bronckers *et al.*, 1984, Bronckers and Wöltgens, 1985). Briefly, the first step consisted of extraction in 10% (weight/volume, w/v) trichloroacetic acid (TCA), a treatment that demineralized the hard tissues, dissolving all $^{45}$Ca and the mineral-associated $^{32}PO_4$ as well as removed non-incorporated and low molecular weight $^{3}$H-activity. Then, the explants were extracted in sequence with distilled water and 10% formic acid (volume/volume, v/v). Both extracts contain amelogenins (Bronckers *et al.*, 1984) and autoradiographic data suggested that these extracts represent amelogenins from different compartments: the water-extractable amelogenins from the extracellular enamel compartment, the formic acid extractable pool from the intracellular compartment (Bronckers *et al.*, 1988). All extracts were counted for $^{3}$H-, $^{45}$Ca- and $^{32}$P-activity, with appropriate standards using a triple label scintillation program. Corrections were made for channel spill-over activity.

**Figures 1a-1d.** Micrographs of the cervical loop region of three-day-old first maxillary hamster molar tooth germs cultured for one day at 6°C (Fig. **1a**), 22°C (Fig. **1b**), 37°C (Fig. **1c**) and 45°C (Fig. **1d**) followed by one day recovery culture period at physiological temperature (37°C). When compared to the control tooth germs (Fig. 1c) cultured at physiological temperature throughout the experiment, the micrographs show that at either low temperature (6°C, Fig. 1a) or at room temperature (22°C, Fig. 1b), these temperatures did not significantly affect the integrity of any of the cells during culture. *In vitro* enamel formation is evident in both cases but appears hypomineralized and the amount deposited is lower than that found in the control teeth (cf. Fig. 1c; see also, **Biochemical results**). Culture at 45°C (Fig. 1d), on the other hand, resulted in serious and irreversible damage and subsequent total degeneration of all cells in the developing tooth germ (arrowheads, Fig. 1d). Also, the ameloblast cell layer has lost its columnar organization and was often detached from the underlying secretory enamel (asterisk, Fig. 1d). At this temperature, there was no evidence of *in vitro* enamel formation in any of the specimens examined (cf. Figs. 1a, 1b and 1c). The arrowheads in Figures 1a-c show the approximate position of the transition between that enamel layer deposited *in vivo* from that deposited during culture *in vitro*. Bar = 40 $\mu$m. A: secretory ameloblast; E: enamel; D: dentin; DP: dental pulp; O: odontoblast; SI: stratum intermedium.

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### Isotopes

1-5-$^{3}$H-proline (specific activity, 27 Ci/mmol), $^{45}CaCl_2$ (specific activity, 1 Ci/mmol) and $Na_3{}^{32}PO_4$ carrier free) were purchased from Amersham International, U.K.

### Statistics

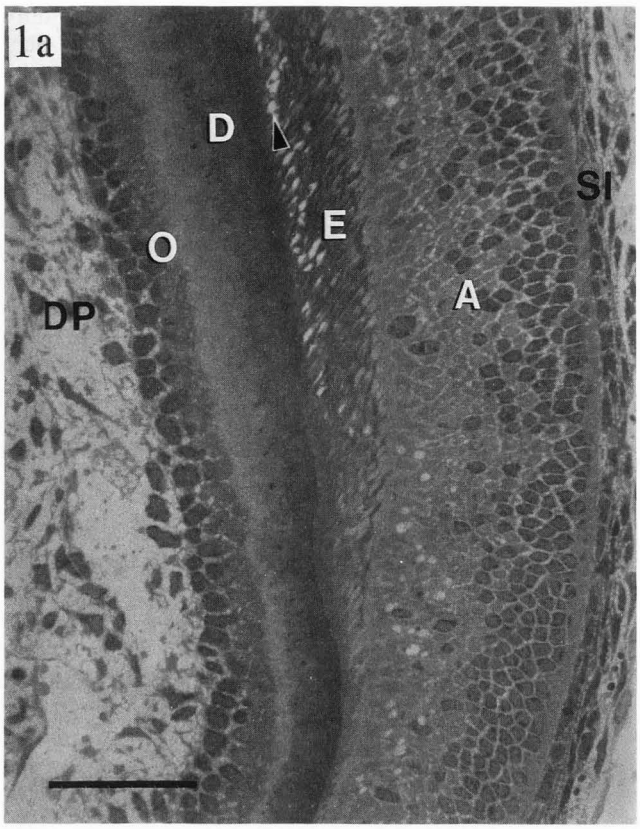
Student's t-test for unpaired samples was used (one tailed).

## Results

### Histological results

Light microscopic results of the tooth germ explants cultured at 6°C, 22°C, 37°C and 45°C for 24 hours followed by a subsequent 24 hour recovery at physiological temperature (37°C) are shown in Figures 1a-d. In the explants grown *in vitro* initially at 6°C, the lowest amount of enamel was deposited *in vitro* during the recovery culture period. But the cells of the enamel organ including the secretory ameloblasts looked healthy and had well developed Tomes' processes (Fig. 1a). In the initial 24 hour culture at 6°C, enamel matrix secretion hardly took place.

Explants grown at 22°C for the first 24 hours and then for 24 hours at 37°C, deposited more enamel *in vitro* than those cultured at 6°C but the long axes of the secretory ameloblasts had shortened to about the same extent as the extra thickness of the *in vitro* deposited layer of enamel (Fig. 1b). No clear accumulation of


1a
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SI
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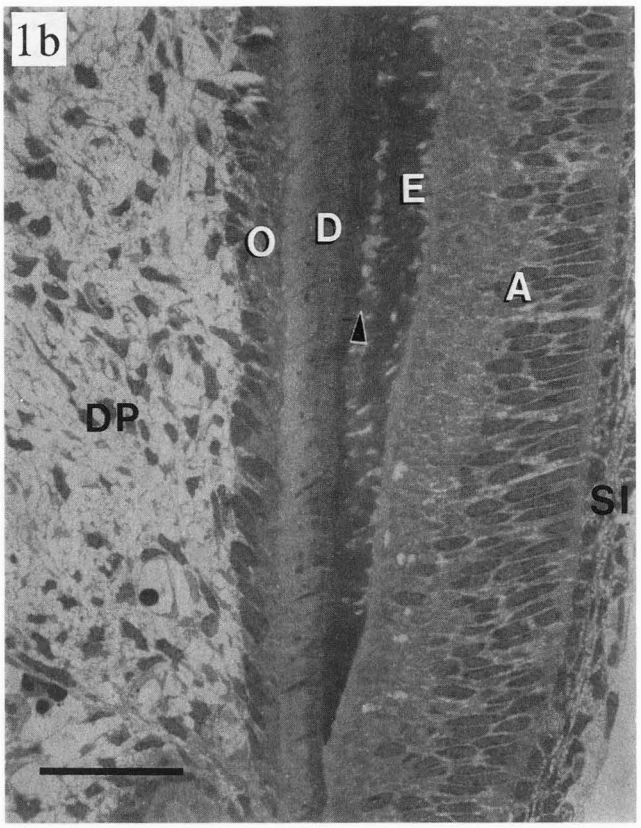
DP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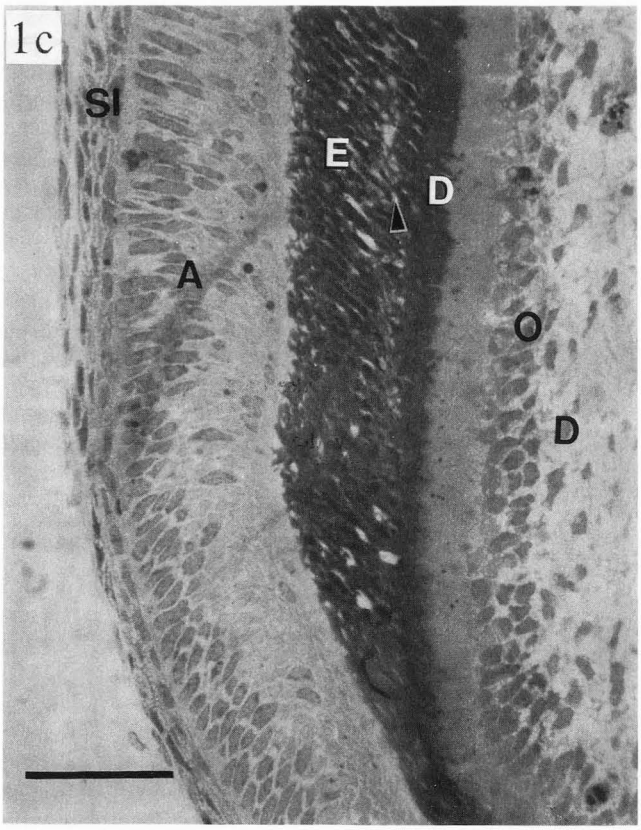
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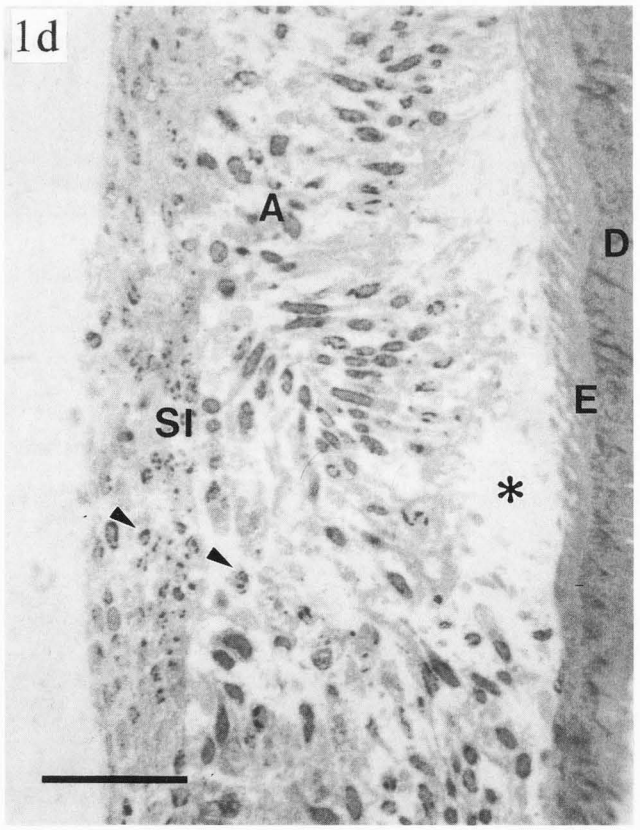
1d
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matrix was found in the cell bodies of the secretory ameloblasts. The specimens belonging to this group had recovered completely from the temperature-induced effects incurred during the initial low temperature culture period.

The thickest enamel layer deposited *in vitro* was encountered in tooth germs grown at 37°C (Fig. 1c). As was the case with the germs cultured at 22°C, there was also an inverse relationship between the length of the ameloblasts and the thickness of the enamel layer deposited during culture. Odontoblasts, on the other hand, were less well developed than those grown at the two lower temperatures, demonstrated by a reduction in the long axes of their cell bodies and a tendency to depolarize.

The most profound temperature-induced effects were observed in the explants cultured for 24 hours *in vitro* at 45°C (Fig. 1d). All the cells were degenerative and contained pycnotic nuclei. This histological picture was already apparent before the onset of the recovery culture period (data not shown). The enamel contained hypomineralized areas and some enamel matrix between degenerated ameloblasts and pre-cultured enamel seemed to have been lost as suggested by the reduction in staining intensity with dilute toluidine blue.

## Biochemical results

**Effect of temperature on dry weight and matrix synthesis** The dry weights of explants grown for 24 hours *in vitro* at the three lowest temperatures did not significantly differ from each other; only the explants cultured at the highest temperature had significantly lower values (Fig. 2).

When the explants were cultured for another day but now all at 37°C, the dry weight values of the explants precultured at the three lowest temperatures were lower than 24 hours earlier, but did not differ significantly from each other. Explants grown the first day at 45°C, however, did not show any change in dry weight when cultured for another 24 hours at 37°C (Fig. 2).

The amount of low-molecular weight $^{3}$H-activity ("TCA-soluble fraction") extracted after the first day of culture at various temperatures was significantly lower at 37°C in comparison with the two lower temperatures and was almost zero at the highest temperature (Fig. 3).

When the explants were exposed to various temperatures without any radiolabels at the first day of culture, and were allowed to recover at 37°C in the presence of the radiolabels on the second day of culture, the TCA-soluble $^{3}$H-activities at the three lowest temperatures were similar, having the same value as the explants grown and labelled the first day at 37°C (Fig. 3). The TCA-soluble $^{3}$H-activity of explants, cultured initially for 24 hours at the highest temperature, however, remained almost zero at the second day of culture (Fig. 3).

In a similar manner, two amelogenin-rich pools, one soluble in formic acid (Fig. 4) and the other in water (Fig. 5) were also examined. The formic acid-soluble $^{3}$H-activity extracted after the first day of culture (putative intracellular amelogenins) showed a gradual increase with increasing temperature but was zero at the highest temperature (Fig. 4). Explants labelled at the highest temperature showed no $^{3}$H-activity in the formic acid extracts. After the second day of culture, the $^{3}$H-values in formic acid extracts were high in explants grown the previous day at the lowest temperature and gradually decreased in explants cultured the previous day at higher temperatures except for the highest temperature that remained almost zero (Fig. 4).

Most $^{3}$H-activity was recovered from the water-extracts (putative extracellular amelogenins) (Fig. 5). When the radiolabels were given the first day of culture, the highest value in the water extracts was found at 37°C, whereas at the lowest and the highest temperatures no label was recovered (Fig. 5). When explants were labelled the second day of culture and the temperature kept at 37°C, explants precultured at the three lowest temperatures all had similar values, but lower than that obtained the previous day at 37°C (Fig. 5). The explants exposed to the highest temperature the first day were not able to recover the second day of culture (Fig. 5). These data, thus, indicated that at low temperature, synthesis and secretion of amelogenins were low but resumed to almost normal levels when temperature was restored to 37°C. At the highest temperature, however, all cells failed to recover biosynthesis and secretion in organ culture.

**Effect of temperature on mineralization** Exposure of tooth germs to various temperatures also affected the uptake of mineral ions. During the first day of culture the uptake of $^{45}$Ca was low at the lowest temperature but increased about three times between 6°C and 37°C; thereafter, it increased twice (Fig. 6a). When the label was given the second day of culture in the recovery period, uptake values were the same as the uptake at 37°C the first day of culture (Fig. 6a).

The uptake of mineral-associated $^{32}$P-activity (TCA-soluble) generally showed a similar pattern, except that between the two highest temperatures the increase was less than the uptake of radio-calcium (about 30%) (Fig. 6b).

Fig. 6c shows how the uptake ratio of $^{45}$Ca and $^{32}$P is related to changes in culture temperature. A small decrease was seen from 6°C to 22°C; after that the uptake ratio slightly increased again. At the highest temperature, the ratio significantly increased from 2 to almost 3.5.

During the recovery period (the second day of culture), the explants precultured at the three lowest temperatures did not differ from each other and had the same value as the explants grown the first day at 37°C, independently from the preculture temperature. The explants grown during the first day at the highest temperature did not recover during the second day at 37°C and had values, similar to the uptake ratio at the first day (Fig. 6c).

## Discussion

The present data illustrate that tooth germs in the secretory stage of amelogenesis synthesize and secrete the highest amounts of proline-rich amelogenins at physiological temperature (i.e., 37°C) but that tooth germ explants can be maintained (for periods of upto 24 hours) at temperatures below physiological values, i.e., at 6°C and at 22°C without clear histological changes or any cell degeneration. When after culture at low temperature explants are brought to physiological temperature, normal tooth development resumes without histologically discernible harmful effects. The labelling data with $^{3}$H-proline show that at low temperatures the biosynthetic activity is strongly decreased but returned to normal levels when the temperature is restored to physiological values. The most rapid rise in biosynthesis and secretion of amelogenins occurs between 22°C and 37°C, an interval in which $^{3}$H-activity in the water extracts is enhanced by a factor of 9.

Previous data suggested that formic acid soluble $^{3}$H-activity constitutes an amelogenin-rich pool obtained from the ameloblasts whereas the water-soluble $^{3}$H-activity reflects the extracellular pool of amelogenins (Bronckers *et al.*, 1988). With these assumptions, we interpret the biosynthetic data as follows: at the lowest temperature the uptake of radiolabelled proline by the cells is mainly determined by the maximal intracellular levels of proline, the resultant of its uptake from the medium and its utilization in the biosynthetic pathway. Since at the lowest temperature examined, the biosynthesis of amelogenins is virtually zero, there is no demand for radiolabelled proline and the maximum amount of this amino acid will be present in the cells. As biosynthesis of proline-rich amelogenins increases with increasing culture temperature (22°C and 37°C) (illustrated by the higher levels of water and formic acid soluble $^{3}$H-activity) the demand for $^{3}$H-proline increases, which explains why at those temperatures the TCA-soluble $^{3}$H-activity is lowered. The virtual absence of low-molecular weight $^{3}$H-activity at the highest temperature, when all cells are necrotic, furthermore implies that the uptake and retainment of proline by the cells are processes controlled by cellular activity; the fact that at the lowest temperature still substantial $^{3}$H-activity is present but not at the highest temperature, also suggests that these processes seem to operate even at the lowest temperature.

Our data also show that if tooth explants have been grown *in vitro* at temperatures lower than physiological temperature, they resume nearly normal development when brought to 37°C. During a recovery period at physiological temperature, however, the deposition of $^{3}$H-labelled amelogenins in the extracellular enamel space did not attain the same high values as explants grown the first day *in vitro* at that temperature. This discrepancy may be associated with a premature degradation of radiolabelled amelogenins, accumulated in the extracellular compartment as suggested by the decrease in formic acid extractable $^{3}$H-activity. Enhanced intracellular degradation of amelogenins has been reported to occur after impairment of secretion by microtubule inhibitors *in vivo* (Nanci *et al.*, 1987). Another explanation could be the presence of protease activity extracellularly (Denbesten and Heffernan, 1988) or a decreased secretion of amelogenins due to aging of the cultivated tooth germ on the second day.

The highest temperature examined in this study (45°C) had a very strong negative effect on the secretory ameloblasts: histologically all cells died within 24 hours, illustrated by the almost complete inhibition of biosynthesis of the proline-rich amelogenins and their secretion to the enamel space. Necrosis and loss of the superficial layers of enamel just below the ameloblastic layer likely accounts for the reduction in the total dry weight of these explants. These processes were irreversible as shown by the inability of these explants to resume biosynthetic and secretory activity on the second day of culture when temperature was brought down to 37°C.

Transepithelial transport of mineral ions toward the mineralizing enamel has been suggested to be under control of the secretory ameloblasts (Bawden, 1989). Aoba and Moreno (1991) proposed that enamel crystal formation is modulated by amelogenins in the tightly controlled environment of the enamel compartment. Absence of newly synthesized $^{3}$H-labelled amelogenins and loss of (preculture) amelogenins from the extracellular compartment in combination with loss of cellular control of the transepithelial calcium transport by the necrotic ameloblasts may account for the increased uptake of particularly calcium ions by the preculture enamel crystals of the explants grown at 45°C. As at the two lower temperatures investigated, there was no histological evidence for any damage to the secretory ameloblasts (also suggested by the reversibility of biosynthesis and secretion of amelogenins). Thus, in this context, it can be assumed that at these temperatures, the uptake of $^{45}$Ca (and the rate of enamel crystal formation) is still under cellular control. In conclusion, these data suggest that developing tooth germs can be maintained for at least 24 hours at lower than physiological temperatures ($\geq 6 \leq$ 22°C) without loss of viability.

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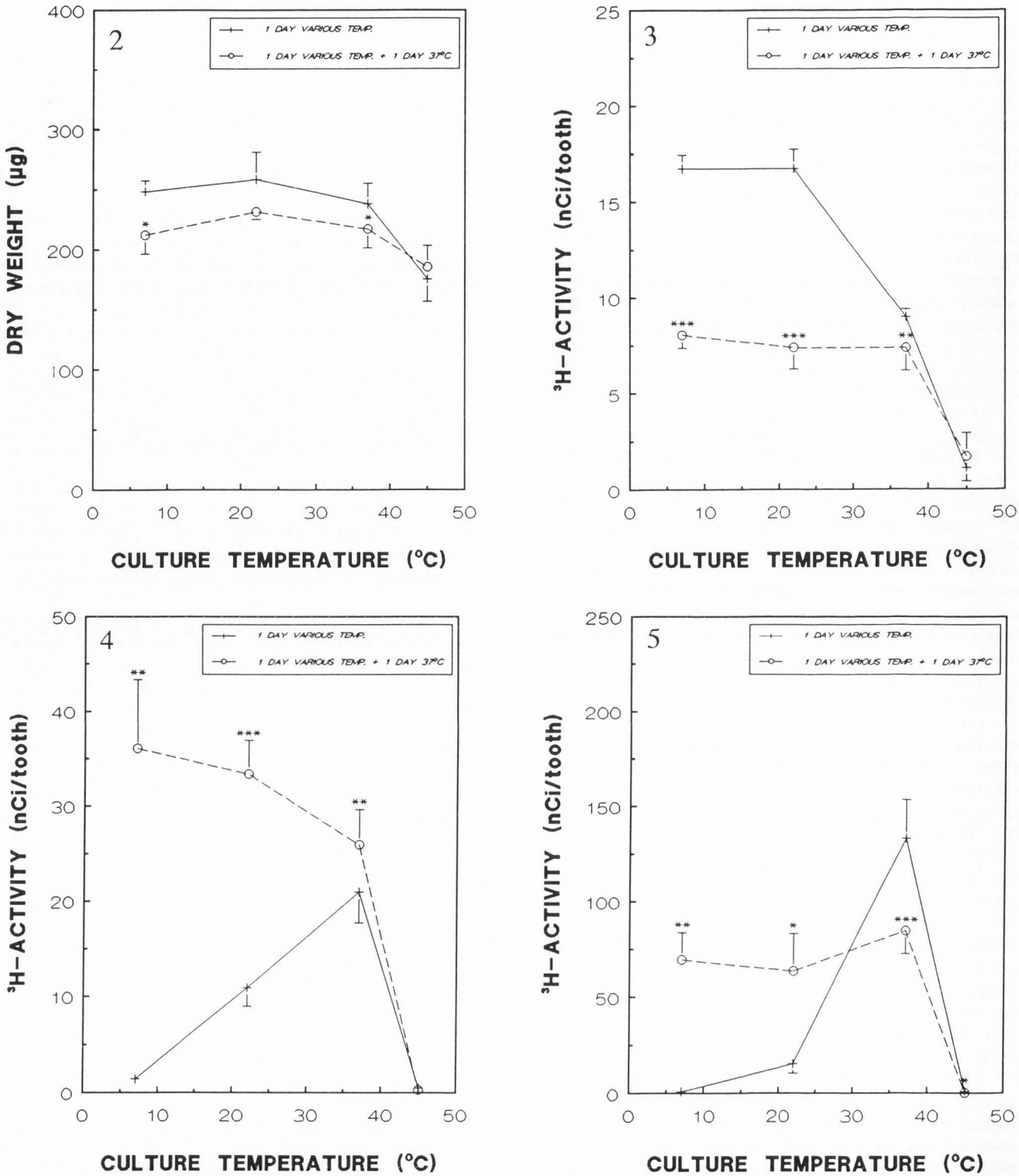


**Figures 2-5.** Effects of culture temperature on the tooth dry weight (**Figure 2**), low molecular $^3$H-activity in tooth explants (TCA-extract) (**Figure 3**), putative intracellular $^3$H-amelogenins in tooth explants (formic acid extract) (**Figure 4**), and putative extracellular $^3$H-amelogenins in tooth explants (water extract) (**Figure 5**) after one day culture at experimental temperature (experimental, +———+) and after a subsequent one day recovery at 37°C (37°C, o---o). Mean and standard deviation (SD), n = 3 explants; * $p < 0.05$ for experimental versus 37°C.

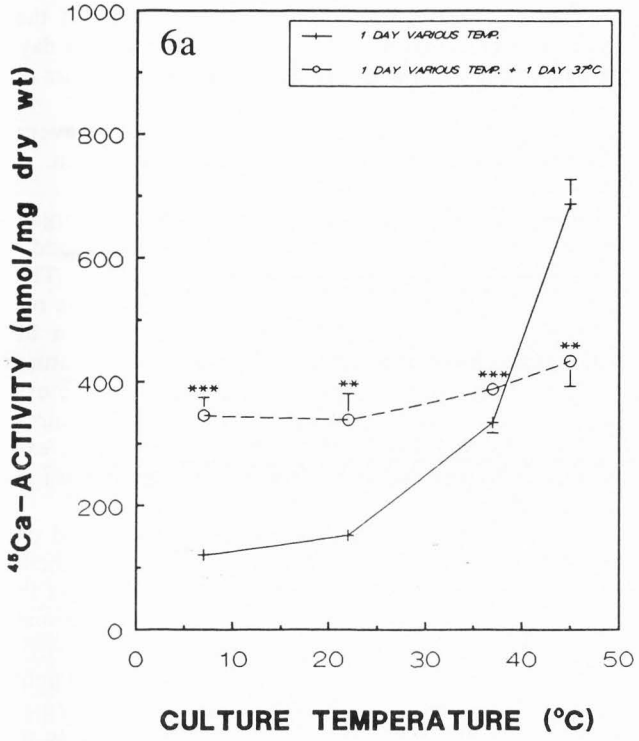


**Figure 6a**. Effect of culture temperature on the uptake of $^{45}$Ca-activity (TCA-soluble fraction) in tooth explants after one day culture at experimental temperature (experimental, +———+) and after a subsequent one day recovery at 37°C (37°C, o---o). Mean and SD, n = 3 explants; ** $p < 0.0015$ and *** $p < 0.001$ for experimental versus 37°C.

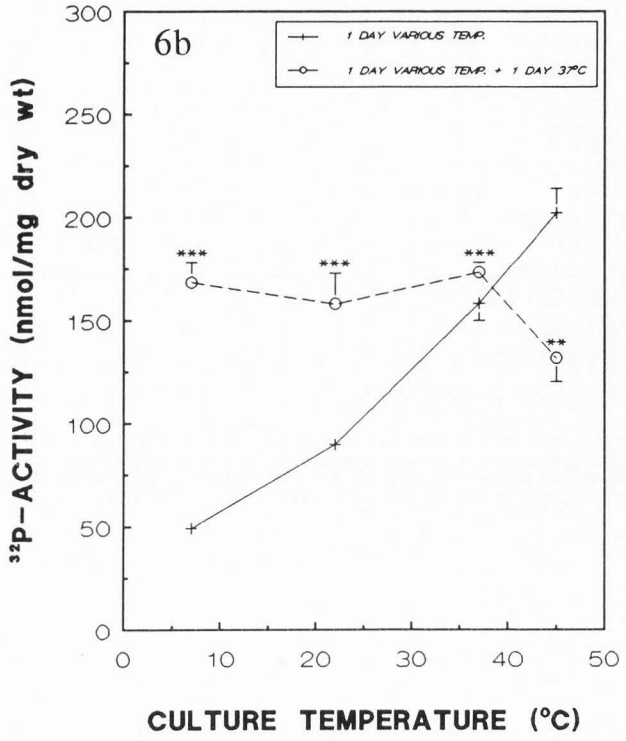


**Figure 6b**. Effect of culture temperature on the uptake of $^{32}$P-activity (TCA-soluble fraction) in tooth explants after a one day culture at experimental temperature (experimental, +———+) and after a subsequent one day recovery at 37°C (37°C, o---o). Mean and SD, n = 3 explants; ** $p < 0.01$ and *** $p < 0.0001$ for experimental versus 37°C.

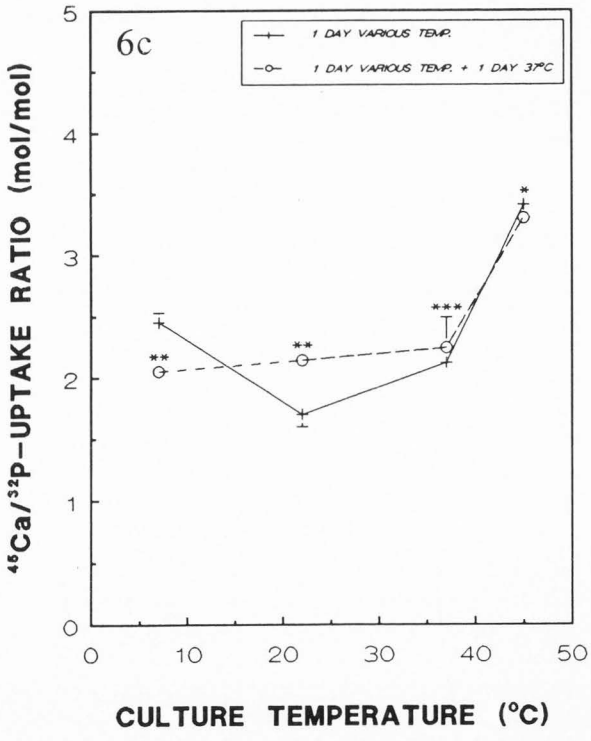


**Figure 6c**. Effect of culture temperature on the $^{45}$Ca/$^{32}$P uptake ratio (TCA-soluble fraction) in tooth explants after a one day culture at experimental temperature (experimental, +———+) and after a subsequent one day recovery at 37°C (37°C, o---o). Mean and SD, n = 3 explants; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$ for experimental versus 37°C.

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## Discussion with Reviewers

**D.R. Eisenmann**: The 37°C specimen illustrated in Figures 1c has odontoblasts which are described as less well-developed than those in the lower temperature specimens. Was this a chance occurrence or is there some other explanation?

**Authors**: The better appearance of the odontoblast at low temperature was consistently found, but up to now, we have no real explanation for this phenomena.

**D.R. Eisenmann**: Could the reduction in labelled extracellular amelogenesis after the recovery day of culture be related to a decline in the quality of the organ culture system at this extended time?

**Authors**: Indeed, the reduction in labelled extra-cellular amelogenins could also be explained by aging of the cultivated tooth germ at the second day.

**D.R. Eisenmann**: Why were the dry weights of the lower temperature explants lower after the recovery day of culture than their counterparts grown only 24 hours *in vitro*?

**Authors**: Also, the lower dry weight at the recovery day could be due to *in vitro* aging of the tooth germ.

**Reviewer II**: The authors, using hamster tooth organ culture, attempted to examine effects of culture conditions on cellular viability and amelogenin secretion. The temperatures tested were 6, 22, 37, and 45°C. The results showed that optimal synthesis and secretion of amelogenins were attained at physiological temperature (37°C). The highest temperature was lethal to the explants (as anticipated?), while the lower temperatures slowed down the metabolism of the cells (again as expected?). Overall the aim of this manuscript appears to be limited.

**Authors**: Upto now, no study has been performed on the effects of various temperatures on tooth development *in vitro*. To do *in vitro* experiments, one first needs an organ culture system with which one can obtain normal mineralization. This is difficult, especially for *in vitro* mineralization of enamel. Only after the improvement of our organ culture technique upto the *in vitro* formation of normal enamel at 37°C, it became possible to study the effect of other temperatures on tooth enamel. Therefore, we disagree with the referee that the aim is limited, because this is the first time that the effects of various temperatures *in vitro* on an isolated tooth organ *in vitro* can be studied and that it could be demonstrated that various parts of a developing tooth can react differently on various temperatures *in vitro*.