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Original Article

Localization of ornithine decarboxylase in mouse teeth. An *in vitro* and *in vivo* study

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ABSTRACT Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, and thus in tissue growth and development, has been localized in mouse dental tissues, *in vivo* as well as *in vitro* by light and electron microscopic autoradiography with radiolabeled α-difluoromethylornithine ([^{3}H]DFMO). Mandibular first molar germs from day-18 fetuses were incubated *in vitro* in the presence of [^{3}H]DFMO and processed for autoradiography. For ODC localization *in vivo*, 3-day old puppies received [^{3}H]DFMO by injection. As controls, puppies were injected either with unlabeled DFMO, or with cycloheximide before administration of isotope. Kidneys and mandibles were excised and processed for autoradiography. *In vitro*, labeling was found in all cell types of the tooth germ, but with a more intense labeling in ameloblasts and odontoblasts. In both these, radioactivity decreased from the tip of the cusps to the cervical loop. *In vivo* the binding of [^{3}H]DFMO in cells of the ameloblast and odontoblast lineages, respectively, showed a gradual increase from the posterior end of the incisor to its anterior end. The distribution of radioactivity in the kidney was in accordance with findings by others. Both the kidney and tooth cell labeling decreased strongly after cycloheximide treatment. The results show that ODC is expressed in tooth-forming cells, and that ODC is not only present in differentiating cells but occurs at higher amounts in mature, secreting cells. The findings suggest that polyamines have a central role in tooth development.

KEY WORDS: *ornithine decarboxylase, polyamines, tooth development, odontoblasts, ameloblasts*

Introduction

The polyamines, putrescine, spermine and spermidine, are normal cellular constituents. Although their physiological function is still not well defined at the molecular level, many studies have shown that their concentration is highly regulated and that normal cellular growth and differentiation require polyamines (Fozard *et al.*, 1980; Heby, 1981; Slotkin *et al.*, 1983; Tabor and Tabor, 1984; Löwkvist *et al.*, 1987).

Activity of ornithine decarboxylase (ODC), the rate limiting enzyme in the polyamine biosynthetic pathway, has been detected in tissues from different species including mammals (Pegg and William-Ashman, 1981). In many cases, the basal activity of the enzyme has been shown to be low in resting and non-dividing cells. On the other hand, marked increases in ODC activity, as well as a rapid accumulation of polyamines, occur in tissues when growth and differentiation are induced, for example during embryogenesis, in response to trophic stimuli, as well as during tumor progression (Snyder and Russell, 1970; Bardocz *et al.*, 1984; Dorn *et al.*, 1985; Löwkvist *et al.*, 1985; Pegg, 1988; Seiler *et al.*, 1990).

Development of an individual tooth, odontogenesis, is governed by a series of sequential and reciprocal epithelial-mesenchymal interactions, leading the tooth from its initiation through morphogenesis to ultimate cytodifferentiation (Ruch and Karcher-Djuricic, 1975; Kollar, 1981; Ruch *et al.*, 1983; Lumsden, 1988). The embryonic tooth consists of two main interacting tissues, the epithelial enamel organ and the mesenchymal dental papilla. The inner dental epithelial cells (preameloblasts) of the enamel organ differentiate into a layer of ameloblasts which secrete enamel matrix. Mesenchymal cells of the dental papilla differentiate into odontoblasts, which produce dentin. These events occur according to a tooth-specific, temporo-spatial pattern (Ruch, 1987). In the molar, terminal differentiation of ameloblasts and odontoblasts is triggered at the tip of the principal cusp and progresses radially towards the cervical and intercuspal regions. The continuously growing incisor of rodent has the advantage over the molar of offering a continuous gradient of cytodifferentiation, progressing from its posterior end to its anterior end, even in adult animals.

The developing tooth is a frequently employed model in which developmental processes and biomineralization are studied. During later years, the mechanisms involved have been elucidated in some detail. To our knowledge, however, no studies have been

Abbreviations used in this paper: [^{3}H]DFMO: α-difluoromethylornithine; ODC, ornithine decarboxylase; HBSS, Hank's balanced salt solution.

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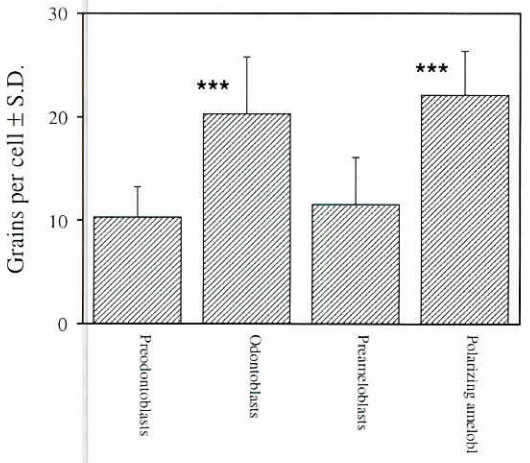


Fig. 1. Grain-counting *in vitro*. *Quantitative autoradiography showing the number of grains per cell in first mandibular tooth germs incubated in vitro in the presence of [³H]DFMO. The number of silver grains per cell was significantly (p< 0.001; n= 33-53) higher in polarizing ameloblasts and odontoblasts at the tip of the principal cusp than in preameloblasts and preodontoblasts at the cervical loop of the anlage.*

done concerning the role of polyamines and ODC during tooth development. The only related existing information concerns ODC activity in bulk pulpal tissue from rat incisor (Rath and Reddi, 1978).

As a first step in elucidating the role of polyamines in tooth development, we have determined the distribution of catalytically active ODC in tooth-forming cells by autoradiography in light as well as electron microscopy, using radiolabeled α-difluoromethylornithine ([³H]DFMO), an enzyme-activated irreversible inhibitor of ODC (Metcalf *et al.*, 1978).

Results

In all autoradiographs, both from the *in vitro* and the *in vivo* specimens, only very few background grains were seen in the resin, outside the tissue. Furthermore, in the controls treated with unlabeled DFMO, grains were virtually absent. Thus, unspecific background was considered negligible.

In vitro autoradiography

In histological preparations of day 18+1 tooth germs, incubated in the presence of [³H]DFMO, silver grain densities were above background (Fig. 4A) in enamel organ cells (preameloblasts, polarizing ameloblasts, stratum intermedium, and stellate reticulum), preodontoblasts, functional odontoblasts, and pulpal cells. The silver grains were distributed over the cytoplasm and nuclei of both proliferating (Fig. 4B) and post-mitotic cells (Fig. 4A,C). This radioactivity decreased significantly ($p<0.001$) from the top of the main cusp to the cervical loop of the molars, i.e. in the ameloblast cell lineage from polarizing ameloblasts to preameloblasts, and in the odontoblast lineage from functional odontoblasts to preodontoblasts (Fig. 1). Very weak labeling occurred in predentin (Fig. 4A,C). No labeling was evident in the control group of germs incubated with unlabeled DFMO (not shown). After heat-inactivation of ODC, the labeling was absent in the cells, but did not decrease in predentin (not shown).

In vivo autoradiography

Catalytically active ODC was localized by light microscopic autoradiography after pulse-labeling newborn mice with [³H]DFMO. Autoradiographic preparations of mouse kidney, labeled with [³H]DFMO (Fig. 4D), showed clearly more silver grains over proximal convoluted tubules than over the renal corpuscules, the distal convoluted tubules, and the collecting tubules, respectively. The renal medulla displayed a weak labeling (not shown). The proximal and distal convoluted tubules from mice treated with cycloheximide, a protein synthesis inhibitor, 5 h prior to [³H]DFMO injection, showed a marked decrease of labeling.

Frontal histological sections of mandibles through the incisor, from mice pulse-labeled with [³H]DFMO, showed a heavy labeling in predentin and dentin (Figs. 2C, 4E). Radioactivity was above background (Figs. 2C, 4E) in all cell types. The number of silver grains gradually increased from the forming posterior end of the incisor (Fig. 2A) to its mature anterior end (Fig. 2C), both in cells of the odontoblastic lineage ($p<0.001$) as well as in cells of the ameloblastic lineage ($p<0.01$) (Fig. 3). Cycloheximide treatment caused a marked decrease ($p<0.0001$) in labeling intensity in the cells but not in the extracellular compartments, including predentin and dentin (Figs. 2D,3). In sections from mice injected with unlabeled DFMO, labeling was virtually absent (Fig. 2B).

In the electron microscopic autoradiographs, grains due to background radioactivity were virtually absent, with the exception of two alpha tracks found in two of the sections. Silver grains were found in predentin/dentin as well as over the nucleus and cytoplasm of cells of the ameloblastic and odontoblastic lineages (Fig. 5A,B). In cell nuclei, ODC was present both in the nucleolus and in the nucleoplasm (Fig. 5B). In predentin and dentin, silver grains were seen both over odontoblast processes as well as over the extracellular matrix (Fig. 5C). A total loss of labeling was evident in the cells, but not in the extracellular compartments, after cycloheximide treatment (Fig. 6A). Labeling was completely absent in sections from mice injected with unlabeled DFMO (Fig. 6B).

In both light and electron microscopic autoradiographs, mandibular bone cells, i.e. osteoblasts and osteocytes, as well as osteoid, were found to be labeled with [³H]DFMO (Fig. 5D). The labeling was less dense in these cells than in the tooth-forming cells, but no quantification was attempted.

Discussion

In this investigation, the distribution of catalytically active ODC was demonstrated by an autoradiographic method, using radioactive DFMO, a specific inhibitor of the enzyme. ODC is the only cellular macromolecule that becomes labeled, when [³H]DFMO is administered (Zagon *et al.*, 1983). DFMO is accepted by ODC as a substrate and becomes decarboxylated by the enzyme, yielding a highly reactive intermediate, which alkylates ODC at its active site. The enzyme thus becomes irreversibly inactivated (Metcalf *et al.*, 1978). There is a stoichiometric attachment of the compound to the enzyme; all molecules of the enzyme that are inactivated become labeled (Pritchard *et al.*, 1981).

DFMO administration *in vivo* represents, for obvious reasons, a physiologically more correct situation than *in vitro* but may, on the other hand, suffer from certain disadvantages. If relatively low concentrations of DFMO are used *in vivo*, there is a possibility that DFMO entry into some cells might occur less extensively than into others, causing artifactual results because of more complete ODC

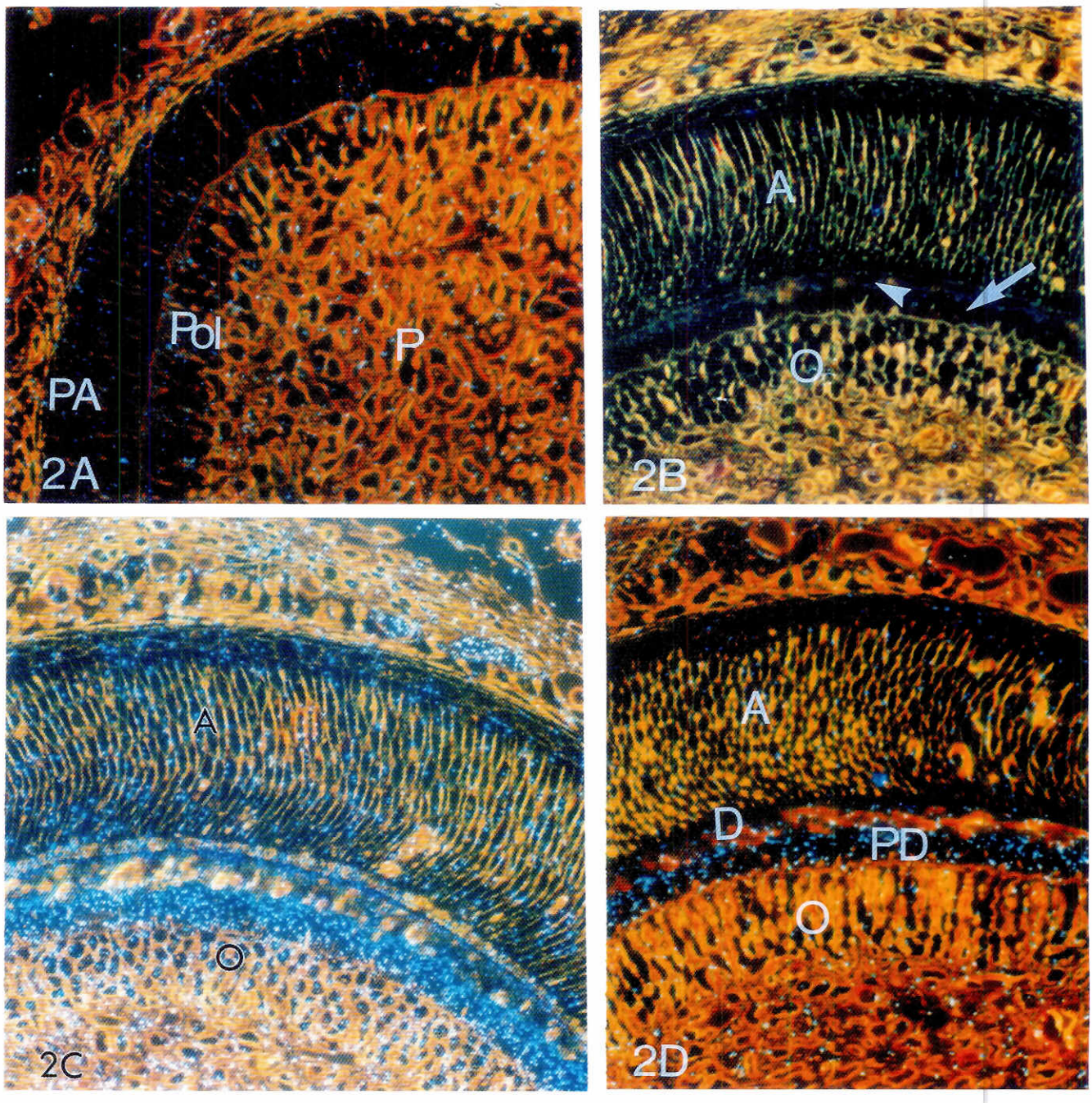


Fig. 2. Autoradiographs of lower incisors from 3 day-old mice *in vivo*. *Frontal section at the posterior end of incisor from a mouse pulse-labeled with [^{3}H]DFMO. A very weak ODC activity was present in preameloblasts, polarizing odontoblasts and preodontoblasts* **(A)**. *Section of lower incisor from mouse treated with unlabeled DFMO (negative control)* **(B)**. *Frontal section at the anterior end of incisor from a mouse pulse-labeled with [^{3}H]DFMO showing numerous silver grains representative of active ODC in ameloblasts and odontoblasts. Predentin and dentin were heavily labeled* **(C)**. *Cycloheximide pretreatment 5 h before injection of [^{3}H]DFMO caused a considerable reduction in labeling in ameloblasts and odontoblasts; the labeling was only somewhat reduced in predentin and dentin* **(D)**. *A, ameloblasts; O, odontoblasts; D, dentin; PD, predentin; PA, preameloblasts; Pol, polarizing odontoblasts; P, pulp; Arrow head, dentin; Arrow, predentin. Dark field images. x560.*

labeling in cells with higher concentrations of DFMO (Zagon *et al.*, 1983). One remedy to this is to give a DFMO dose high enough as to inactivate virtually all the enzyme in the tissue. The dose used here, 1 mg/kg body weight, is that used by Zagon *et al.* (1984) for this reason. The risk can be further ruled out by *in vitro* experiments, where well-defined, higher doses of DFMO can be used. In the present studies, the labeling pattern in dental tissues was found to be the same *in vivo* and *in vitro*.

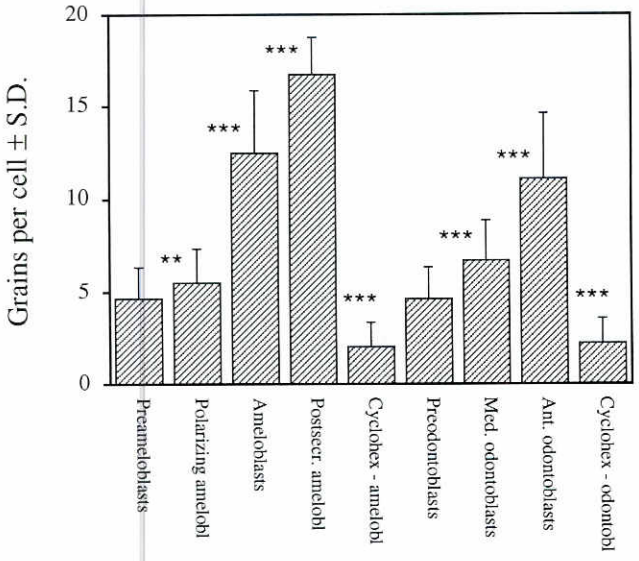


Fig. 3. Grain-counting *in vivo*. *Number of grains per cell in serial sections of lower incisors from mice, pulse-labeled with [^{3}H]DFMO, and from mice pretreated with cycloheximide before isotope injection. The number of silver grains in cells of the ameloblastic and odontoblastic lineages increased from the posterior, forming end of the incisor to its anterior end. The highest grain density was seen in differentiated cells of the anterior end of the anlage (post-secretory ameloblasts, ameloblasts and odontoblasts). After cycloheximide treatment, a considerable decrease in cell labeling occurred over odontoblasts and ameloblasts. Statistical significances are denoted in the diagram (***p< 0.001; **p< 0.01; n= 39-50). The significances refer to the difference between each bar and the bar to its left. In the case of the cycloheximide-treated specimens, in contrast, the statistical testing refers to a comparison between these with ameloblasts and odontoblasts, respectively.*

It was found that [^{3}H]DFMO-labeled ODC in kidneys from pulse-labeled newborn mice was confined to tubule cells in the cortical region, and that the strongest labeling occurred in the proximal convoluted tubules. This is in accordance with previous immunohistochemical and autoradiographic findings by others (Persson *et al.*, 1982; Zagon *et al.*, 1984; Dorn *et al.*, 1985; Koibuchi *et al.*, 1993), thus demonstrating the accuracy of the methodology. Furthermore, the substantial reduction in radiolabelling in the kidneys and tooth-forming cells after *in vivo* cycloheximide treatment provides further evidence of the validity of the method. ODC is known to have an extremely rapid turnover rate, implying that the enzyme activity disappears when protein synthesis is inhibited (Russell, 1980). This is an additional confirmation that the labeling did in fact represent ODC localization.

In the *in vivo* study, the extracellular dentin, predentin, and enamel were heavily labeled. In predentin and dentin, the electron microscopic autoradiographs showed silver grains over odontoblast processes as well as in the matrix. Silver grain density was not reduced after cycloheximide treatment, though the drug caused a marked decrease of radiolabelling in cells. Moreover, predentin was only weakly labeled in tooth germs incubated with [^{3}H]DFMO and chased with unlabeled DFMO *in vitro*. This weak predentin labeling was not lost after heat-inactivating the enzyme. Taken together, these findings strongly suggest that the labeling of enamel and dentin/predentin was due to nonspecific absorption of labeled DFMO, the mechanism of which is not understood.

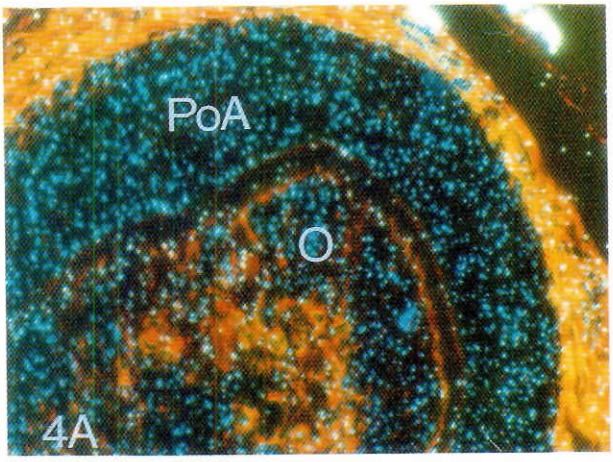
The presence of ODC in cell nuclei has been a matter of debate. Our autoradiographic results give further support for earlier biochemical, autoradiographic and immunocytochemical findings of ODC in nuclei (Heby and Emanuelsson, 1981; Emanuelsson and Heby, 1982, 1983; Anehus *et al.*, 1984; Zagon *et al.*, 1984).

The level of ODC/polyamines has been shown to regulate growth and development of a variety of tissues and organ systems (Russell and McVicker, 1972a; Löwkvist *et al.*, 1983; Slotkin *et al.*, 1985). Our findings show that catalytically active ODC is not only present in tooth-forming cells but also that in these cells, it is distributed according to a developmentally regulated pattern. Additionally, we confirmed by autoradiography the presence of ODC in bone cells, such as osteoblasts and osteocytes. This was demonstrated earlier by biochemical assays (Rath and Reddi, 1978; Löwik *et al.*, 1986).

Most earlier studies were focused upon the occurrence and role of polyamines during cellular proliferation and differentiation, for example at early stages during embryogenesis. In such systems, the initially high levels of polyamines and ODC activity decrease when these processes cease. It was therefore a somewhat unexpected finding that both the highly differentiated and active odontoblasts and ameloblasts displayed higher levels of enzyme than did the dividing and differentiating cells of the odontoblast and ameloblast lineages, respectively. This may be looked upon in the perspective of studies by others, who found evidence that polyamines are presumably also involved in normal functions of specialized cells and tissues, in addition to their rather well-established roles in processes of cell growth and proliferation. For example, pancreas (Rosenthal and Tabor, 1956), mammary gland (Russell and McVicker, 1972b), myelin (Seiler and Schmidt-Glenewinkel, 1975) and pituitary gland (Harik and Snyder, 1974) have been reported to contain high concentrations of polyamines. Based on findings like these, it has been suggested that polyamines have specific roles in generation, storage or exocytosis of secretory material (Seiler and Heby, 1988).

Ameloblasts and odontoblasts undergo morphological and metabolic changes during tooth development, and the differentiated odontoblasts and ameloblasts play a central role in the formation of dentin and enamel, respectively. Odontoblasts synthesize and secrete the constituents of the organic matrix of dentin, including collagen type I, dentin phosphoprotein, decorin

Fig. 4. Autoradiographs showing the distribution of ODC in mouse tissues. *In day 18+1 lower first molar (principal cusp)* **(A,B,C)** *silver grain density was above background over tooth-forming cells. Predentin was weakly labeled. PoA, polarizing ameloblasts; O, odontoblasts. Dark field image x560* **(A)**. *High magnification view (x1400) at the cervical loop of the molar showing silver grains in both cytoplasm and nuclei of undifferentiated preameloblasts (PA) and preodontoblasts (PO); arrow, mitotic figure* **(B)**. *High magnification view (x1400) showing the presence of numerous silver grains in differentiated odontoblasts (O) and polarizing post-mitotic ameloblasts (PoA). Predentin (Pd) shows weak labeling. Si, stratum intermedium* **(C)**. *Dark field image (x560) of kidney cortex from a [^{3}H]DFMO pulse-labeled mouse, showing extensive labeling of proximal convoluted tubules (P) and less activity over renal corpuscules (c) and distal convoluted tubules (D)* **(D)**. *High magnification view (x1400) showing the distribution of ODC in a frontal section at the anterior end of incisor from a mouse pulse-labeled with [^{3}H]DFMO. A, ameloblasts, D, dentin, PD, predentin, O, odontoblasts* **(E)**.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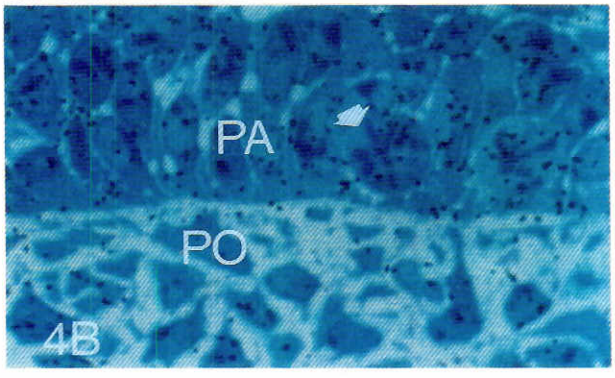
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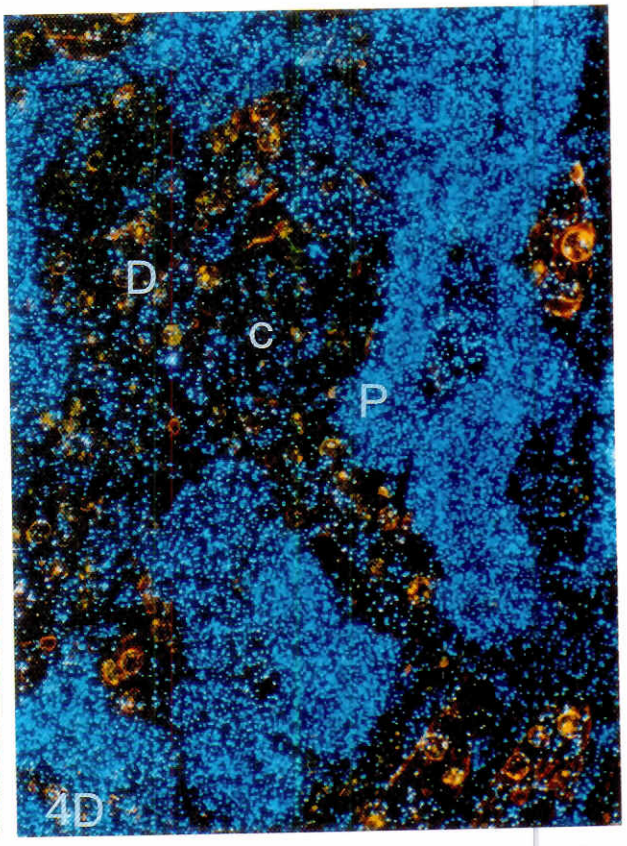
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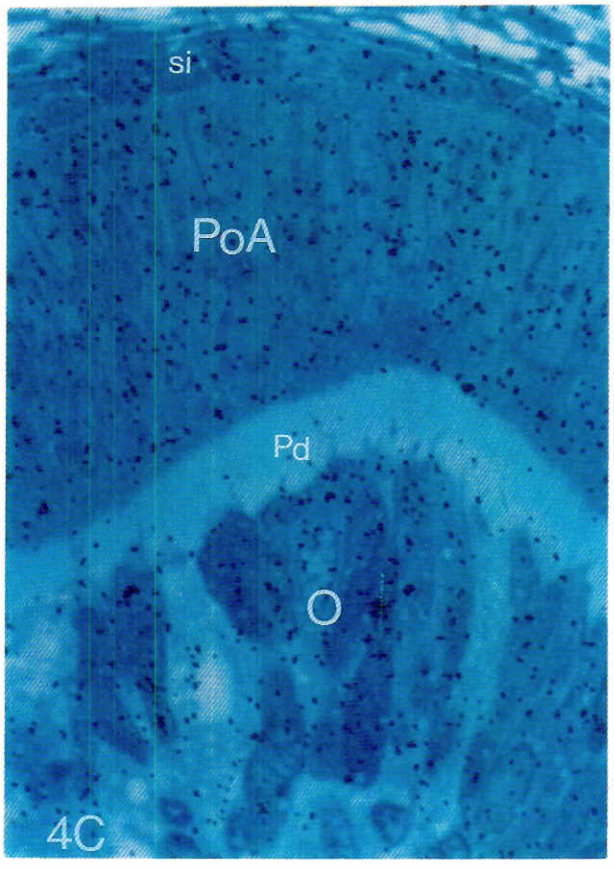
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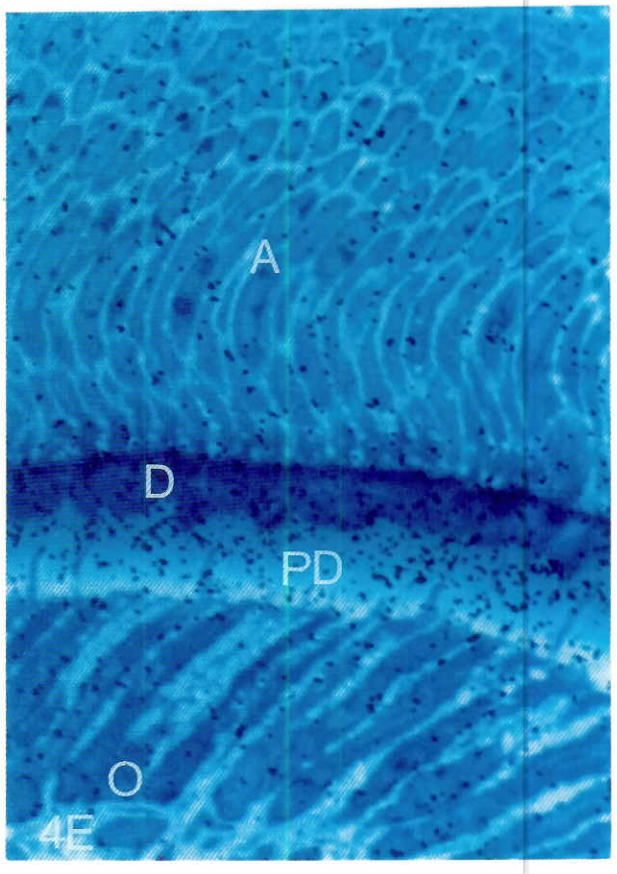
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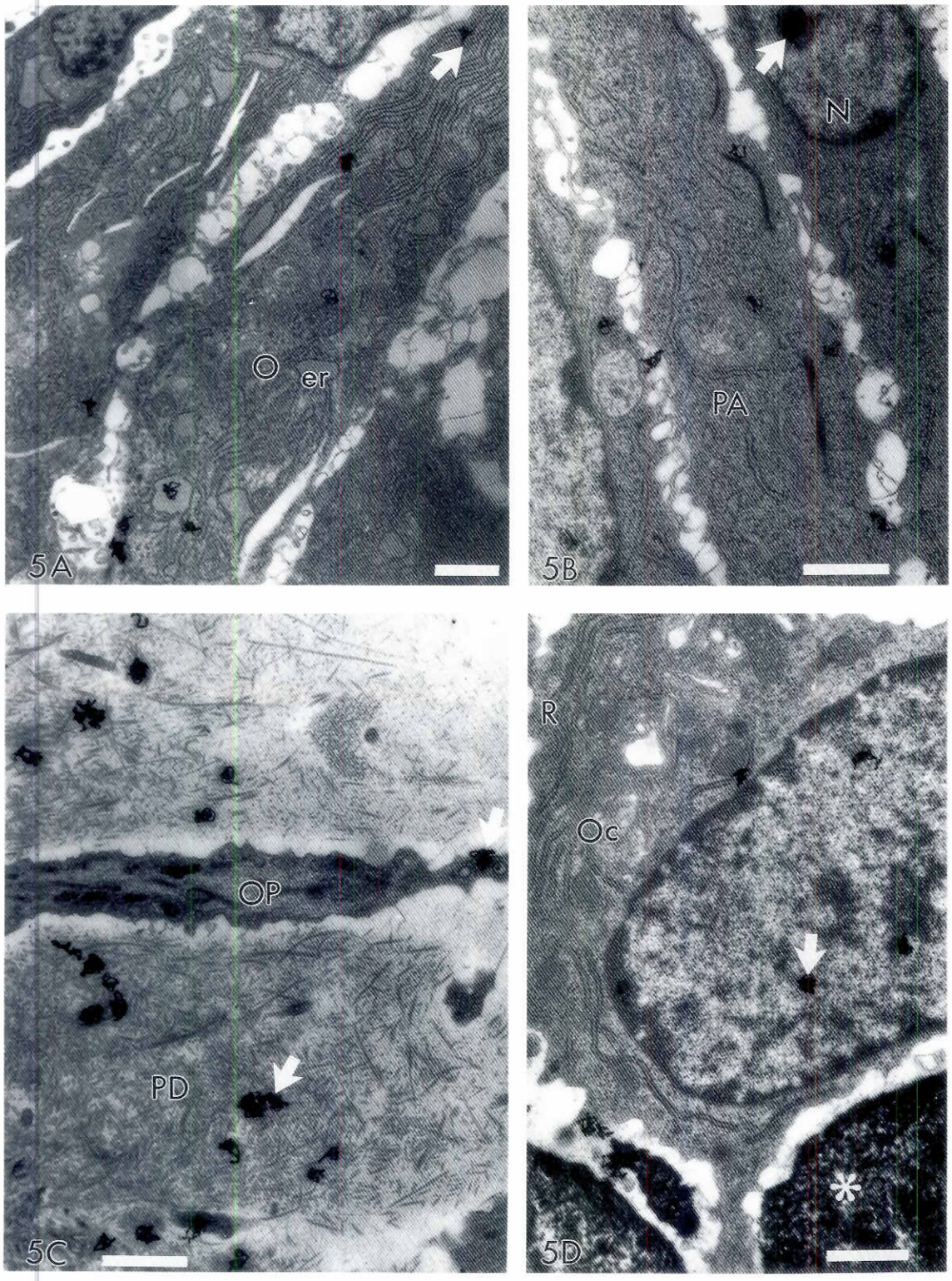

Fig. 5. Electron microscopy autoradiographs of lower incisors from 3-day old mouse, pulse-labeled with [^{3}H]DFMO. *Silver grains, representative of ODC, were visible in the cytoplasm as well as in the nuclei of ameloblasts, odontoblasts and osteoblasts* **(A,B,D)**. *In the nuclei, silver grains were seen both in the nucleolus and in the nucleoplasm* **(B,D)**. *In predentin, the labeling was evident in the collagenous matrix as well as in odontoblast processes* **(C)**. *O, odontoblasts; PA, polarizing ameloblasts; N, nucleus; er, endoplasmic reticulum; PD, predentin; OP, odontoblast process; Oc, young osteocyte; asterisk, bone; arrows, silver grains. Scale bars represent 1 μm.*

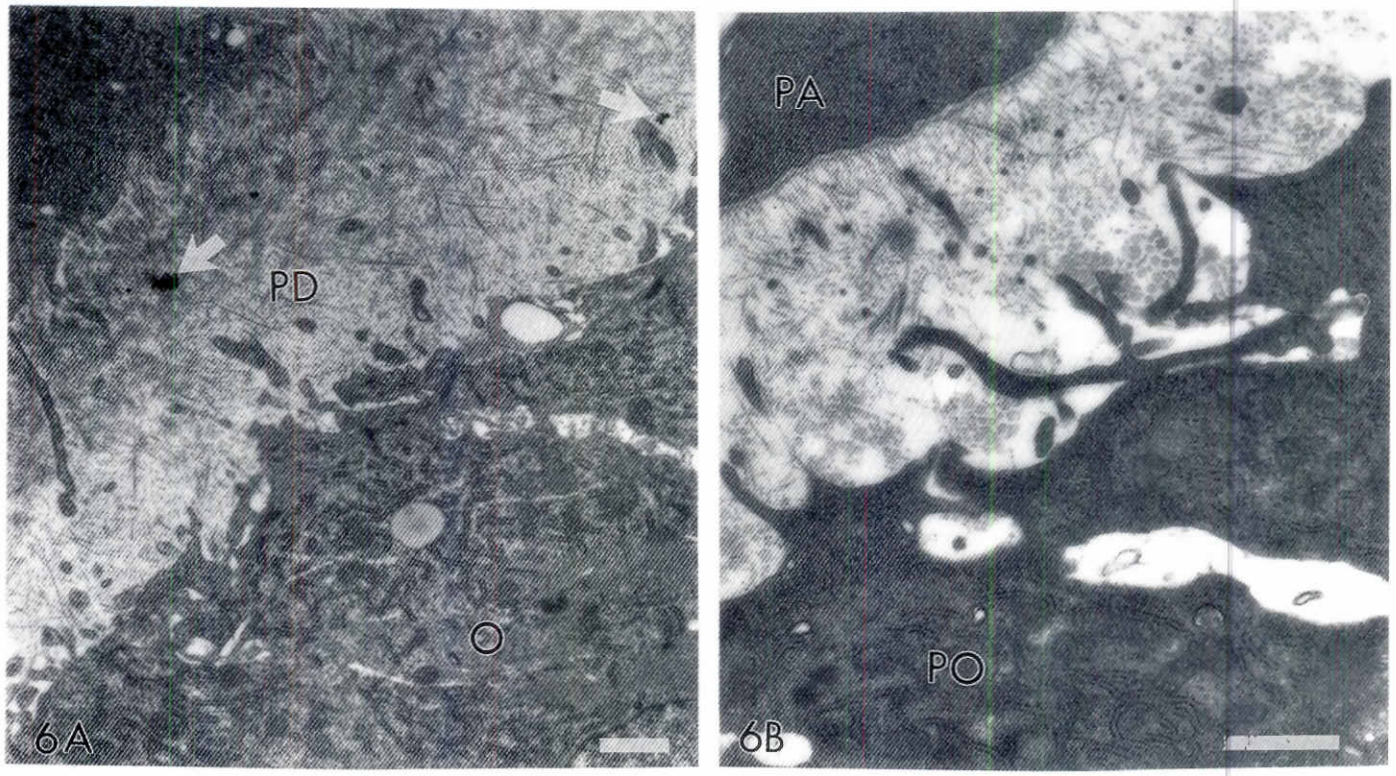

Fig. 6. Electron microscopy autoradiographs from 3-day old mice. *Cycloheximide treatment induced a total loss of labeling in the cells but only a slight reduction in the extracellular matrix* **(A)**. *A total absence of labeling was seen in control preparations from mice injected with unlabeled DFMO* **(B)**. *PA, preameloblasts; PO, polarizing odontoblasts; PD, predentin; O, odontoblasts; arrows, silver grains. Scale bars represent 1 μm.*

proteoglycan, and other non-collagenous proteins. In addition, this cell is actively involved in the transport and regulation of inorganic ions, such as calcium ions, constituting the hydroxyapatite mineral phase of dentin (Linde, 1992). Furthermore, the odontoblasts also reinternalize some matrix constituents from predentin. The high activity of ODC in dentinogenically active odontoblasts suggests that polyamines may have a regulatory function on such processes during dentinogenesis.

Secretory ameloblasts are high, columnar, polarized, epithelial cells, which synthesize and secrete enamel proteins, such as amelogenins and enamelins. Also in this case, the polyamines may be of importance. Post-secretory ameloblasts are actively reabsorbing enamel matrix material as part of the process that makes the enamel attain its full mineralization. The even higher ODC activity in these cells thus suggests the importance of polyamines in transport processes (cf. kidney).

In conclusion, the presence of ODC in tooth-forming cells suggests that polyamines play an important role in odontogenesis. ODC is clearly present in differentiating cells, but shows an even higher activity in these cells, when they have reached their fully mature, secretory stage.

Materials and Methods

In vitro localization of ODC

Swiss mice were mated *inter se* and the females were checked daily for a vaginal plug, the appearance of which was considered as day 0. Lower first molar germs were dissected out from day-18 fetuses and cultured at 37°C in the presence of 5% CO_2 in air for 24 h in Transwells (Costar Europe, Badhoevedorp, The Netherlands), containing RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 μg/ml kanamycin, and 180 μg/ml ascorbic acid. After 24 h, the germs were incubated for 30 min in a fresh medium containing 3 μM [^{3}H]DFMO (specific activity: 25.13 Ci/mmol, NEN Du Pont, Stockholm, Sweden), then chased 3 times for 30 min with the same medium as above but containing 5 mM unlabeled DFMO. After this, the germs were rinsed with Hanks' balanced salt solution (HBSS), containing 5 mM DFMO and fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 -7.4. The specimens were dehydrated in graded alcohols and embedded in Agar 100 (Agar, Stansted, Essex, England).

Two types of controls were made. Tooth germs were cultured in a medium containing 3 μM unlabeled DFMO. Alternatively, germs were incubated in hot water at 90 °C in order to heat-inactivate the enzyme before incubation with isotope.

Semi-thin sections (1 μm) of each specimen were coated with Ilford K5 nuclear emulsion for light microscopy autoradiography and stored in black boxes at 4°C for 3 weeks.

In vivo localization of ODC

Three-day old newborn mice were used. Two littermates were pulse-labeled with 40 μCi [^{3}H]DFMO injected intraperitoneally. After adding unlabeled DFMO, the final specific activity of the isotope was 3792 Ci/mole. In total, the mice received 1 mg/kg body weight DFMO as used by Zagon *et al.* (1984). Two puppies, serving as controls, received 1 mg/kg body weight unlabeled DFMO in saline via i.p. injection. Finally, two other littermates received a single i.p. injection of cycloheximide (20 mg/kg body weight) 5 h before the administration of 40 μCi [^{3}H]DFMO. After 30 min the animals were killed by decapitation, the kidneys and mandibles were

removed and washed in HBSS, then fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2-7.4. The specimen were further post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer, pH 7.2, dehydrated in graded alcohols and embedded in Agar 100. Semithin sections were processed for light microscopy autoradiography as described above. As for mandibles, frontal serial sections of the jaws were made through the incisor from its posterior, forming end to its anterior, mature end.

Evaluation of light microscopy autoradiograms

After 3 weeks exposure time, the *in vitro* and *in vivo* autoradiographic preparations were developed in Kodak D 19, fixed in 15% $Na_2S_2O_3$ and stained with Richardson's azure II-methylene blue. The distribution of radiolabeling was quantified by visual counts of silver grains at x1000 magnification in non-adjacent sections over longitudinally sectioned cells. The counting was done on sections from three levels of the incisor: 1) the posterior end of the anlage which contains preameloblasts, and the dental papilla containing preodontoblasts and polarizing odontoblasts; 2) the median level, containing polarizing ameloblasts, odontoblasts and predentin; 3) the anterior end of the incisor containing ameloblasts, odontoblasts, predentin/dentin and, sometimes, enamel. Silver grains over post-secretory ameloblasts at the maturing anterior end of the anlage were also evaluated. In the molars, the quantification of cellular radiolabelling was assessed in tooth-forming cells from the top of the principal cusp to the cervical loop and the intercuspal zones. Statistic evaluation was performed using Student's *t* test.

Both for the *in vivo* and the *in vitro* study, background was estimated by grain counting over the resin outside tissue sections in radiolabeled specimens, and over the tissue sections from the control specimens, treated with unlabeled DFMO.

Electron microscopic autoradiography

Ultrathin sections from *in vivo* specimens, as described above, were collected on formvar-coated grids and stained with uranyl acetate (5% aqueous solution) and Reynolds' lead citrate. The sections were coated with three protective carbon layers and then with Ilford L4 liquid nuclear emulsion, using a loop technique. After exposure for 10 weeks at 4°C in the dark, the autoradiographs were developed in Kodak D19 and fixed in 15% $Na_2S_2O_3$. The preparations were then examined in a Philips 400T electron microscope at 60 kV.

The experimental outline, as described above, was approved by the Animal Research Ethics Committee at Göteborg University.

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