Autoradiographic and Biochemical Investigations of Collagen Biosynthesis And Participation in the Dentine Extracellular Organic Matrix.* (32470)

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Interest is rapidly increasing in the detailed localization and molecular organization of fibrous proteins in tissues at different stages of development, and during various physiological conditions. In young developing rodent molar primordium, the formation and organization of dentine collagen might reflect, in part, the biosynthetic characteristics of the individual odontoblast. One of these characteristics is related to the intracellular synthesis and export of tropocollagen units into an extracellular environment with a subsequent provision related to condensation sites for the growth of hydroxyapatite crystals. Approximately one quarter of the amino acid residues of dentine collagen are either proline or the closely related imino residue, hydroxyproline, found characteristically in collagen (1,2). No hydroxyproline is found in the adjacent enamel organic matrix protein(s) (3). Several autoradiographic studies have traced the utilization of isotopic proline in developing rodent molars(4,5). However, biochemical studies of collagen biosynthesis by the merocrine-like secretory odontoblasts and, further correlations with autoradiographic kinetics of a specific isotopic precursor, have not been reported.

Methods. Rats of the Wistar strain, 5 days old, were randomly selected from 12 litters for this study. All animals received a single intraperitoneal injection of 2 μc C^{14}-Proline (μ/l, specific activity, 202 mc/mM, New England Nuclear Corp.) in 0.1 ml isotonic saline.

Autoradiography. Four animals per time period were sacrificed by decapitation at intervals ranging from 15 minutes to 24 hours. These tissues were immediately placed in Bouin-Hollande solution for fixation and embedded in nitro-cellulose-paraffin. Longitudinal (mesiodistal) sections of the maxillary first molars were cut at 5 μ. Autoradiographs were prepared by the dipping technique using Eastman Kodak NTB2 emulsion and were developed after exposures ranging from 10-30 days(6). After photographic fixation and washing, the PAS stained specimens were counter-stained with Harris' hematoxylin for histological examination.

Biochemical and radiochemical analyses. Animals were decapitated at times ranging from 15 minutes to 24 hours following administration of isotopic proline. The maxillary first molars were carefully dissected from each animal, weighed wet, and pooled in groups of 30 teeth per time period. The sample size was 159-164 mg per time interval. Maxillary first molars were extracted in 8 ml of 1 M NaCl in a constant shaking device for 48 hours at 4°C. They were then centrifuged at 18,000 rpm for 1 hour and the supernatant was dialyzed against distilled water for 48 hours. The residue was extracted twice with 4 ml of 0.3 M TCA at 90°C for 30 minutes and the combined extracts centrifuged for 1 hour. The supernatants were dialyzed, dried, and hydrolyzed with 2 ml of 6 N HCl at 130°C for 90 minutes. After hydrolysis, the nitroso imino acids were prepared following the method described by Myhil and Jackson(7). The nitroso imino acids were separated on columns containing Beckman spherical resin (type AA-15), operated at 55°C and using pH 3.28 in 0.2 N citrate buffer. The Beckman amino acid analyzer model 120 B was used to automate this procedure as previously described by our laboratory(8). For detection of radioactivity the effluent was passed through a 1.0 ml flow cell in a Nuclear-Chicago scintillation spectrometer at a flow rate of

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50 ml/hour. The coupling of these methods offered a sensitive system for detection of small amounts of radioactivity in the effluent. A recovery of 95-100% was observed of the N-nitroso imino acids placed on the columns.

Results and discussion. The dentine and enamel matrices were well preserved in the unerupted molar tooth of the 5-day-old rat. The majority of our autoradiographic observations were made on the cusps of the maxillary first molars because of the relative permanency of these structures during the experimental period. The distribution of proline-C^{14}, in the molar during the 24 hours following administration of the isotope are illustrated in Fig. 1. Fifteen minutes after administration of proline-C^{14}, a weak but definite labelling of the odontoblastic cytoplasm could be seen. By 4 hours following isotope administration, the cytoplasmic grain density had been exported to the extracellular predentine (arrow) and appeared as a distinct band of radioactivity in the dentine organic matrix. After 24 hours, the grain density remained discrete, but further displaced away from the secretory cell site. In contrast to the dentine, a diffuse grain density appeared throughout the enamel matrix. Microscopic observations revealed significant reactivity in both the amelogenic and dentinogenic systems of the developing rodent molar. Reactivity in the dentine collagen represented both proline and hydroxyproline, whereas only proline was assumed to be incorporated into the enamel protein(s). No significant grain density, above background, was observed throughout the dental pulp. Therefore, it is reasonable to assume that chemical results obtained from homogenized whole tooth primordia reflected only utilization of labeled proline and its various metabolic products. No reference will be made to the proline incorporation into the ameloblasts and enamel organic matrix protein(s).

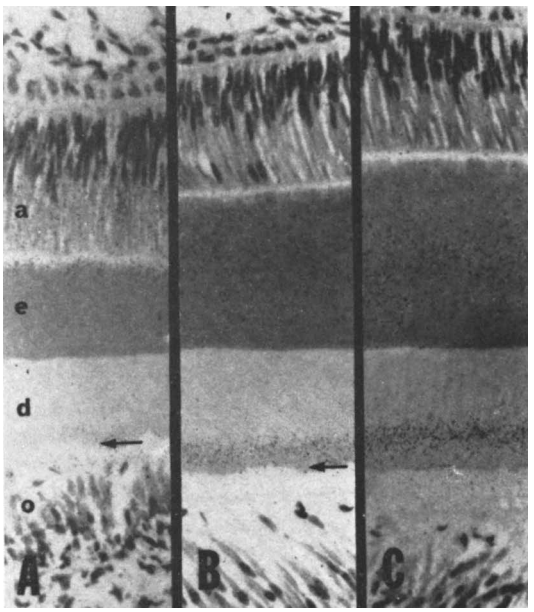


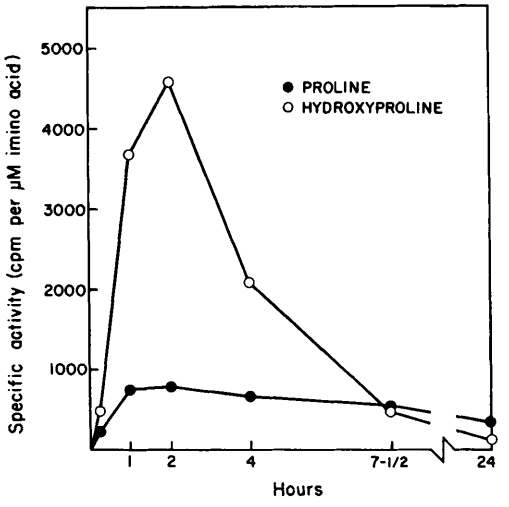
FIG. 1. Autoradiographs demonstrating proline-C^{14} uptake in the odontogenic apparatus of rat molar cusps. Magnification: × 360. A. Fifteen minutes after administration of the label, the reaction is seen over the cytoplasm of the ameloblasts (a) and odontoblasts (o) B. Four hours after administration, the reaction over the intracellular regions is reduced, whereas that in the predentine, dentine (d), and enamel matrices (E) is more intense. C. At 24 hours after administration of the labelled material, little or no reactivity is discernible intracellularly. The label appears to diffuse throughout the enamel matrix, whereas in the dentine it appears as a discrete band adjacent to the predentine-dentine junction (arrow).

In Table I the concentrations of proline and hydroxyproline in the experimental rodent molar are presented. There was approximately 24-fold more proline than hydroxyproline found in the neutral salt fraction, reflecting the presence of large amounts of soluble noncollagenous material. Hydroxyproline in the salt soluble collagen fraction reached its maximum specific activity 2 hours following administration of the labelled precursor (Fig. 2). These chemical findings, and the autoradiographic observation of intracellular localization of grain density during the first 4 hours following isotopic administration, further suggested that the synthesis of tropocollagen was a major metabolic function in the developing molar tooth. The rapid appearance of the label in hydroxyproline re-

TABLE I. Relative Concentrations of Proline and Hydroxyproline in 5-Day Old Rat Molar Teeth.

	μM amino acid per gram wet weight of tissue	
Fraction	Proline	Hydroxyproline
Soluble	14.60 (± .67)*	.587 (± .13)
Insoluble	25.10 (± 2.92)	16.00 (± 2.25)

* Standard deviation of the mean.



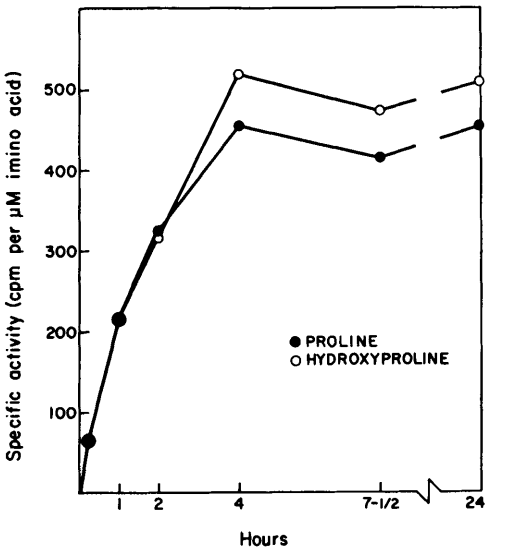


FIG. 2. Graphic representation of specific activities of proline and hydroxyproline in the salt soluble fraction following administration of 2 μc proline-C^{14} in 0.1 ml isotonic saline.
FIG. 3. Graphic representation of specific activities of proline and hydroxyproline in the insoluble fraction following administration of the labelled material.

flected the fast rate of collagen biosynthesis. Apparently, the isotopic material required 4 hours to be incorporated into soluble collagen and migrate through the odontoblastic cytoplasm into the extracellular dentine matrix. The appearance of the detectable label at 4 hours in dentine coincides with the peak in specific activity of the insoluble collagen, as well as a sharp drop in the radioactivity present in the soluble collagen. This substantiates the assumption that the collagen which reaches the dentine area becomes rapidly insolubilized. We assumed, therefore, that the collagen in the TCA extraction was predominately insoluble, dentine matrix fibers. The significantly lower specific activity of proline in the soluble fraction reflected the multi-functional metabolic pathways of intracellular proline, such as conversion to glutamic acid, as well as its incorporation into the many non-collagenous proteins (resulting in isotope dilution).

Both autoradiographic and chemical methods demonstrated that the intracellular synthesis of dentine collagen occurred at a very rapid rate ($T\frac{1}{2} = 2$ hours) (Fig. 1, 2). Both imino acids reach their respective, maximum, specific activities by 4 hours (Fig. 3). Within the insoluble collagen fractions throughout the period of this experiment, a 1:1 ratio between the specific activities of both imino acids was evident. These findings indicated that hydroxyproline in developing dentine collagen was synthesized at a very fast rate. Further, our findings are compatible with the rapid differentiation and growth observed during this stage of development. The fast turnover of hydroxyproline agreed with its rapid appearance in new dentine, and its rapid insolubilization and subsequent detection in the insoluble collagen fraction. Dentine collagen, in this connection, behaved more like bone collagen (Mills and Bavetta, personal communication) than soft tissue collagen where this process of insolubilization occurs at a much slower rate(9).

Summary. The kinetics of proline and hydroxyproline and their respective incorporation into dentine collagen during rapid dentinogenesis has been reported. The incorporation of proline into collagen and the subsequent hydroxylation of this imino acid takes place as an intracellular event, demonstrated by the rapid rise in the specific activity of hydroxyproline in the neutral salt collagen fraction and the intracellular localization of the grain density. Four hours after administration of proline C^{14}, the biosynthesis

of collagen and its export from the odontoblast into the extracellular organic matrix was completed. Both proline and hydroxyproline exhibit similar specific activities in the insoluble collagen fractions throughout the experimental period. The developing rodent molar was found to be an excellent experimental model for the study of rapid collagen synthesis and its role in the formation of the dentine organic matrix.

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Effect of Endogenous Erythropoietin on Replicating Hemopoietic Stem Cells. (32471)

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Cells of most neoplastic tissues fail to differentiate normally. Although elevated levels of erythropoietin have been detected in patients with leukemia(1), inappropriately diminished erythroid differentiation(2) of hemopoietic stem cells leads to anemia in many of them. It remains unclear how the target cell for erythropoietin has become refractory to the hormone that usually induces its differentiation.

We have examined the suggestion that replication, *per se,* might render hemopoietic stem cells refractory to erythropoietin (3). Previous autorepopulation experiments in mice indicated that stem cells in the leg, shielded from a supralethal dose of x-rays, colonize the spleen and synthesize deoxyribonucleic acid (DNA) during the first 5 days after irradiation(4). Injections of human urinary erythropoietin failed to induce erythropoiesis during this proliferative phase(4). We wondered whether the exogenous erythropoietin had been inactivated or if the stem cell had become temporarily refractory to it.

Studies described here were undertaken: (1) to demonstrate by a radiobiological method that replication of stem cells occurs within the first five days after irradiation; and (2) to see whether erythropoietin secretion produced by hypoxia would induce erythroid differentiation in the "shielded mouse" during this postulated proliferative phase.

Methods. Mice. Carworth Farms No. 1, 10-to-12-week-old female mice were used in these studies. They were housed, 8 to a cage, on a bed of sterilized ground corn cobs, and fed Rockland complete mouse diet. Drinking water, provided *ad libitum,* was acidified to pH 2.5 with HCl to control Pseudomonas infections.

Irradiation. Irradiation procedures were identical to those previously described(4). Total-body x-ray, from a General Electric Maxitron, 250 x-ray machine delivering an average output of 60 R/min, was administered to the mice which were confined during irradiation in perforated lusteroid tubes on a

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