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A Study of the Rat Molar Pulp at Various Ages

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A STUDY OF THE RAT MOLAR PULP
AT VARIOUS AGES

by

Rafaël D. Pinzon

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

March

1965

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I dedicate this Thesis to my
wife, Celia, and my daughters,
Ivette, Smella and Yesica.

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CHAPTER I

INTRODUCTION

The rat molar has been an experimental tool of dental research for many years. Numerous investigators have described morphologic similarities between human and rat molars: Schour and Van Dyke (1934), Berman (1957), Kindlova (1963), and others. Kozlov (1959) suggested that the physiology of both human and rat molar pulps is probably similar. Schour and Massler (1962) stated that the histology and physiology of the dental tissues of the rat molar are quite similar to those of human molars, as well as the form and function of both teeth.

Accordingly, a number of histologic studies have used the rat molar as a test object and analyzed its reactions to various medicaments: Miyamoto (1957), Berman (1957), Mansukhani (1959), Kozlov (1959), Kiryati (1958, 1962) and others; and to different conditions: Schour and Van Dyke (1934), Glickman and Shklar (1954), Weider, Schour and Mohammed (1956), Wade (1964) and others.

The results of the aforementioned studies have been interpreted mainly in the light of their practical application through clinical treatment.

Berman (1957) tested the rat molar pulp reaction to different materials and found that his results seemed to correlate well with findings in other species: dog and human pulps tested

with the same medicament (Calcium Hydroxide) by Nyborg (1955). At this time, therefore, the rat molar is the biologic object of choice because of its pointed similarities to the human molar and its availability in large numbers. Moreover, it can be observed and studied under controlled conditions (Berman, 1957).

Thus, the importance of the rat molar as an experimental object of dental research has been repeatedly demonstrated. Although some investigators have described the development of these teeth (Addison and Appleton, 1921 and 1922, Hoffman and Schour, 1940, Dienststein, 1956, and Johannesen, 1961), the literature reveals no complete and detailed descriptive studies of the histological features of the normal rat molar pulp. Only a few of its histological characteristics have been described while comparing the reactions of experimentally treated pulps with untreated control groups: Kiryati (1958), Wade (1964); or when measuring the rate of dentin opposition: Hoffman and Schour (1940). Moreover, the rats used in these studies have been of specific ages.

Therefore, the purpose of this paper is twofold: it is primarily intended to establish an index of cell proliferation in the rat molar pulp at various ages by means of measuring cell frequency of DNA synthesis by an autoradiographic technique. Secondly, an analysis of the histologic anatomy of the normal pulp of the rat maxillary first molar, at different ages and by several histologic methods, will be presented in order to pro-

vide an adequate standard for comparison in future investigations of the rat molar pulp.

CHAPTER II

REVIEW OF THE LITERATURE

A. General Descriptive Features of the Rat Molar Pulp

Schour and Van Dyke (1934) had stressed the difference between the histology of the normal and so-called normal molar of the rat. Signs of degeneration in the pulp of normal molars were related to an observable causative factor; the molar pulp was not affected in the absence of any stimulus.

Hoffman and Schour (1938, 1940) studied the rate of apposition of primary and secondary dentin in 63 albino rats which ranged in age from one to 502 days. Multiple injections of 2.0% alizarine red S were given to the animals at different intervals from two hours to four weeks, prior to sacrifice. They observed that the apposition of primary dentin began at birth and continued to about 135 days of age. After 80 days this growth was limited to the floor and roof of the pulp chamber. The daily incremental rate of dentin apposition ranged from 16 to 2 microns. The rate of apposition decreased according to four gradients: a) Age gradient, from dentinal surfaces to the pulp; b) Locus gradient, from the tips of coronal cusps to apices of roots; c) Spiral gradient, from root surface toward the bifurcation; and d) Anteroposterior gradient, from first to third molars. The incremental pattern was conical, with the apex

toward the cuspal growth center.

As for the secondary dentin, apposition began at 35-40 days of age, when clinical occlusion was initiated, and continued throughout the length of the investigation, which was 500 days. This apposition began earlier and was faster in the pulp horns and the incremental cones became reversed in their pattern with the apex toward the pulp.

The conclusion reached was that dentin growth was not uniform. They also concluded that, "concomitant with the continuous process of attrition, secondary dentin is being apposed and the pulp recedes".

Kiryati (1958) who tested selected medicaments upon lacerated molar pulps of 96 albino rats, ranging in age from 60 to 120 days, also described some aspects of the normal molar pulp. It was found that a great number of morphologic variations may occur and still be categorized as normal. Large and hyperemic blood vessels were seen, as well as capillaries, which appeared scattered in the odontoblastic layer sometimes reaching the predentin. The pulp contained those cellular elements usually found in other connective tissues, i.e. fibroblasts, spindle-shaped mesenchymal cells near the endothelial cells, some histiocytes at some distance from the vessel wall, plasma cells and extravasated erythrocytes. Many pulps were observed to have an unusual arrangement of the odontoblastic layer showing empty spaces between cells, that was attributed to the histologic pre-

paration.

Wade (1964) described some normal characteristics of the molar pulp of albino rats 125 and 132 days of age. The predentin measured nine to ten microns in width at 100 X and was regular in arrangement. It was uniform in thickness and evenly distributed. The odontoblasts appeared regularly arranged in a palisade manner around the periphery of the pulp with the nuclei presenting the usual elongated shape. A sharply defined boundary was described for these cells. He also found that abundant young fibroblasts and other cellular components such as young connective tissue cells were evenly distributed throughout the pulpal area. He did not find any inflammatory exudate nor a difference in the number or size of the pulpal blood vessels in these two age groups.

Kindlova (1963) studied the blood vessels of 50 molars from adult rats and analyzed them under the electron microscope. The main vessel supplying the molar pulp was located in the center of the pulpal tissue, from which minute branches, arose and went toward the periphery of the pulp forming a continuous network. The density of the capillary network seemed to correspond with the density of the odontoblasts forming the outer surface of the pulp adjacent to the dentin. "Multifarious communications" between the main vessel and the peripheral capillaries apparently ensured the co-ordinated blood circulation in the pulp.

B. Age Changes in Connective Tissue

Asboe-Hansen (1963) assumed that progressive changes occur during the aging of any connective tissue. He described a reduction in turgor and thickness in the dermis of old individuals. After analyzing the findings of several authors, he also concluded that the fibrillar density is increased as the connective tissue becomes older.

This generalization on the maturation and aging of connective tissue can be summarized in the following review.

1. Changes in Animal Connective Tissue

Sobel and co-workers (1953, 1954 and 1956) studied the ratio of collagen to hexosamine in selected organs of various animals and humans. Hexosamine was included in the analysis because it is a constituent of mucopolysaccharides, such as hyaluronic acid, and found in free form or combined with protein in the connective tissue ground substance.

Sobel, Zutrauen and Marmorston (1953) analyzed the collagen and hexosamine content in the skin of 120 rats one to three months of age. It was found that during growth the collagen in connective tissue of the rat skin increased in proportion to the body weight and the hexosamine increased almost in a linear manner. Therefore the ratio of collagen to hexosamine increased.

Later, Sobel, Marmorston and Moore (1954) studied the femurs of 57 male and 28 female rats one to three months of age. Although the rate of deposition of collagen was found to decrease somewhat in the growing femur of the rat, the hexosamine-con-

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taining material showed a greater decrease. Thus, the ratio of collagen to hexosamine increased with growth.

Sobel and Marmorston (1956) also showed that during the growth process in connective tissues, from several organs of different animals, the collagen is deposited faster than the mucopolysaccharides which form the ground substance where the fibers are embedded.

Verzar (1956) studied the tendons of the rat tail of animals from a carefully controlled colony in which 50% of the rats reached 23.5 months of age and only a few survived for as much as 31 or even 41 months.

The thermic contraction upon heating the tendons (packets) was absent in three to four months old rats whereas such contractions appeared very marked in the tendons of the old specimens. As for the individual collagenous fiber, the contractile factor was stronger in the older animals than in the younger specimens. Asboe-Hansen (1963) interpreted the findings of Verzar (1956) as an indication of increase of the fibrillar density in the aging connective tissue.

Gross (1950) discussed the predominance of argyrophilic reticular fibers in the newborn rat skin which changed gradually with aging into the classic collagenous fibers. Through electron microscopy he was able to show that the width of the fibers also increased.

2. Changes in Human Connective Tissue

The results obtained by several investigators on aging human connective tissues are very much similar to those previously described for other animals.

Myers and Lang (1946) studied the elastin and the collagen contents in thoracic aortas of autopsy specimens from 83 human beings aged 15 to 88 years. The tunicas media and intima histochemically analyzed showed the elastin content decreased after 60 years of age. The collagen content of the aortas was constant until age 45 when a slow continuous increase was observed throughout the remaining years of life. They concluded that the ratio of collagen to elastin increased with age.

Faber and Moller-Hou (1952) determined the content of collagen and elastin from the complete aortas from 85 normal and hypertensive individuals. The rate of collagen deposition increased with age while that of elastin decreased, confirming the findings of Myers and Lang (1946). They concluded, furthermore, that the total amount of both substances was increased during the aging process. In the proportion, however, the ratio was increased for collagen and decreased for elastin.

Banfield (1955) studied abdominal skin samples from three young fetuses (measuring 2.5 centimeters to 18 centimeters crown-rump), another fetus of seven months, a child three years old, and three adults aged 65, 68, and 85 years. It was found that in general the collagen fibrils increased in width as the

From the foregoing, it can be generalized that the observation by Asboe-Hansen (1963) pointed earlier in this review, regarding the increase of the fibrillar density in the aging connective tissue, is true for both animal and human specimens.

C. Pulpal Changes with Aging

A review of the literature did not reveal many studies of changes that occur normally in the aging pulp. Some isolated information, however, is given by different investigators.

1. Rat Molar Pulp Studies

Hoffman and Schour (1938, 1940) reported that the secondary dentin apposition in the rat molar is responsible for the reduction in size of the pulpal chamber. Berman (1957) studied 122 rats aged 30 to 360 days in which partial and total pulp-tomies were practiced experimentally in order to study the pattern of healing under different medicaments (calcium hydroxide, zinc oxide and eugenol). Marked differences were found between the unoperated molar pulp of younger and older animals, clinically and histologically. The pulp chambers of the old specimens were reduced to a very small size due to secondary dentin growth. They also showed little if any bleeding when the operations were performed. On the other hand, the younger specimens showed large pulps histologically and presented profuse haemorrhage on amputation.

2. Human Studies

Kronfeld (1937) gave a description of the changes occurring in the aging human pulp. The very young pulp of recently erupted teeth was rich in blood vessels and nerve endings, with an intact odontoblastic layer. These observations are occasionally seen in dental pulps of adult individuals, but never in old teeth, where continuity of the odontoblasts is interrupted.

Fibrosis was considered as the most characteristic change observed in the aging pulp. The cells were reduced in number and fibrous connective tissue took its place. The blood vessels and nerves were also reduced, resulting in pulp atrophy, in which the odontoblastic layer was no longer present.

Areas of abnormal calcification were also described with advancing age. Scattered, very small bodies of calcium salts or larger accumulations known as pulp stones were some typical examples of these abnormalities.

Some of the preceding descriptions were confirmed by Provenza (1964) who described an increase of the fibrillar components over the cellular components in the aging human pulps. He considered these changes as normal and compared them to those occurring in connective tissues elsewhere in the body.

It should be noted that the two preceding references did not give detailed descriptions of their methods of evaluation nor of the size or character of their samples. They have stood, however, for the authoritative view of pulpal aging for many years.

Zerlotti (1964) studied the pulp of 55 non-carious teeth (25 from patients 11 to 15 years old, 25 more from patients aged 40 to 55 years and five from individuals older than 70 years). The pulps were removed from the teeth and fixed with a freeze-drying method. A loss of water-soluble tissue components was minimized by denaturing the sections in absolute ethanol.

Hematoxylin and eosin, as well as several special methods of staining, were employed (periodic acid schiff, toluidine blue, silver impregnation, acid fuchsin-picric acid) and certain other histochemical methods of localizing some substances and extracting or digesting others (2,4 - dinitrofluorobenzene reagent, ribonuclease, desoxyribonuclease, 0.1 M citrate buffer, 0.1 M phosphate buffer). The sections treated histochemically were stained by the various methods previously mentioned.

The following changes with aging were observed: collagen fibers increased in number while the argyrophilic fibers and ground substance showed a proportionate decrease; the reticular fibers and ground substance of young pulps were readily dissolved by some enzymes, but the collagen fibers and extracellular matrix of old specimens resisted their actions. The old pulps were also found to be more resistant to the action of buffer solutions, lower in water content and less reactive to chemical agents. The latter was indicated by the collagen that did not react to silver, and by the extracellular matrix that presented a weaker stain with periodic acid schiff, dinitrofluorobenzene

and toluidine blue. In general Zerlotti found great similarities between his results in dental pulps and those findings of other studies utilizing other connective tissues.

D. Autoradiographic Studies of Mitosis in Connective Tissue

1. In Connective Tissue

Messier and Leblond (1960) studied the connective tissue of 22 male Sherman albino rats averaging around 200 grams of weight. The rats were injected intraperitoneally with tritiated thymidine and sacrificed from one hour to 95 days after injection. Hematoxylin-eosin stained sections were autoradiographed with NTB 3 emulsion and analyzed.

They found that most connective tissues showed a moderate frequency of labeled nuclei. Labeled cells were found, among other places, between the muscle fibers of the heart, within the periodontal ligament of the incisors and occasionally in fat deposits. Labeled nuclei were rarely found in the dense connective tissue of the tendons and the sclera. In connective tissue derivatives such as cartilage and bone, which have some similarities in function with that of the pulp, the labeling was found at the growth centers and at the early intervals of sacrifice. Labeled osteoblasts, however, were common along the walls of the mixed spicules of bone.

Schultze, and Oehlert (1959) and Leblond, Messier and Kopriwa (1959) found what they described as "surprisingly" high numbers of labeled cells in connective tissues of mice and rats. Both

studies were concerned with the normal uptake of radioactive material by the cells in several tissues of both species of animals.

Leblond et al. (1959) suggested that the differences in frequency of labeled cell populations in connective tissues was due to different proliferative potencies and therefore, local conditions could determine whether or not an increase in the rate of connective tissue cell mitosis should occur.

2. In Pulp

The labeling reactions in molar and incisor teeth were also briefly described by Messier and Leblond (1960). Labeled cells were very rarely seen in the pulpal tissue of the molar tooth. On the other hand, the incisor contained many labeled nuclei in the area of the tooth insertion in the bone, which is the growth center of the tooth. There, labeling was common in odontoblasts and in the neighboring cells of the pulp.

Hoffman and Gillete (1962) utilized autoradiographic techniques in order to study the DNA synthesis in the pulp and periodontal ligament in a series of hamsters, five to 30 days old. They thus sought to measure the mitotic activity of both pulp and periodontal ligament, during the eruption of a multicrooked tooth.

Their findings showed that "almost all of the mitotic activity in the pulp was concentrated in the apical areas, adjacent to Hertwig's epithelial diaphragm".

CHAPTER III

METHODS AND MATERIALS

Animals

Maxillae and mandibles obtained from seventy male albino rats* were used in the present experiment. Seven groups, of ten animals each, 10, 15, 30, 60, 90, 125 and 400 days of age were selected. They all appeared to be healthy and normal on arrival, and were sacrificed within two days after arrival.

Operations

The animals were injected intraperitoneally with tritiated thymidine**, specific activity 1.90 curies per millimole. The dosage level was 70 microcuries per 100 gr. of weight. Between one and two hours later they were anesthetized with ether and sacrificed by decapitation. This was done since Hughes and co-workers (1958) have found that tritiated thymidine is only available to the cells one to two hours after injection. Moreover, they stated that all labeling occurs within the first hour after the radioactive material has been injected. Following sacrifice, the maxillae and mandibles were dissected and immediately fixed in ten per cent neutral formaline.

* Supplied by Abrams Small Stock Breeders, Chicago, Illinois.

** Supplied by Schwarz BioResearch, Inc., Orangeburg, New York.

Specimen Preparations

The molar regions of both the maxilla and mandible from one side of each animal were decalcified in formic acid*, dehydrated in ascending orders of alcohol (75, 95 & 100%) and embedded in paraffin. Longitudinal sections, four to six microns thick, were obtained by standard laboratory procedures. Only the maxillary sections were studied histologically. The remaining mandibular and maxillary regions of the opposite side and the lower sections of the same side were saved for future studies.

Four methods of staining were employed: Heidenhain's azan stain (modification of Mallory's connective tissue stain) was used to study primarily the collagenous connective tissue fibers in the pulp; Gomori's method of silver impregnation for reticular fibers; alcian blue (pH 2.6) and periodic acid Schiff reactions, for acid and neutral mucopolysaccharides, respectively, according to McManus and Mowry; and Harris' hematoxylin and eosin stains for the general morphology.

Autoradiographs were developed on the same slides used for the hematoxylin and eosin stains following basically the technic used at the Medical Research Center, Brookhaven National Laboratory, Upton, N.Y., and compiled by Emil R. Adamik and published by Schwarz Bioresearch Inc., Orangeburg, N.Y. Some modifications

* Manual of Histologic and Special Staining Technics, published by the Armed Forces Institute of Pathology, Second Edition, 1949, Page 4.

in this technic were devised and will be mentioned later in the method and materials section of this paper. Autoradiographs were used in this study to assay the amount of growth in pulps of different ages as indicated by mitosis.

Autoradiographic Preparatory Technic

Histologic sections were cut at four to six microns and mounted with albumin on the frosted end, standard 3x1 inch glass slides. Specimens were deparaffined just prior to application of the autoradiographic emulsion NTB3*.

Since the NTB3 emulsion required refrigeration storage it was necessary to allow the bottle of emulsion to come to room temperature. It was then placed in a water bath at 43° C. for approximately 15 minutes to change it from a gel to a liquid state. Approximately 40 milliliters were poured into a Coplin jar and one to two drops of Tween 20 (a surface active agent) were added to assure even distribution of the emulsion. The solution was stirred and allowed to stand until the emulsion reached approximately 43° C.

The slides were dipped in the emulsion in the following manner: three slides were placed on a metal warming plate to warm at 43°C. and then placed into the Coplin jar containing the emulsion. Three more slides were placed on the metal warming plate. The first three slides were removed from the emulsion, drained

* Distributed by William A. Sykes, Research Division, Special Products, Rochester, N.Y.

on a paper towel and left standing in a drying rack with the frosted ends up, permitting the emulsion to drain. The preceding steps were timed so that the slides were immersed in the emulsion for approximately ten seconds. The emulsion was then permitted to gel on the slides for five to ten minutes before being air dried with an electric non-heating fan. When dry, the slides were stored in black boxes containing perforated capsules filled with "Dririte" (a drying agent) and the boxes were sealed with black plastic tape. They were then kept in the refrigerator for two weeks.

The autoradiographic emulsion on each slide was developed in the dark room using a Wratten #4 safelight at a distance of four feet. The slides were placed in a standard staining dish, containing Kodak D-19 developer and maintained there for two minutes. They were then rinsed for five to ten seconds and fixed in Kodak fixer for another two minutes. Finally, they were washed for ten minutes and let dry before being placed back in the original boxes.

The original procedure as devised in the Brookhaven National Laboratory was modified in two ways: first, the amount of time that the slides were left in the emulsion was reduced from 15 to 10 seconds, and secondly, the developing process was different than the one described by Adamik. These modifications were found to bring about better results and simplification of the entire technic for the purposes of this study.

Autoradiographic Evaluation Technic

In order to study the numbers of labeled cells in the rat molar pulp at different ages, the six most comparable specimens (animals) were selected from the ten in each group. The major basis for selection was that the sections of each specimen would be similar to the other five specimens in the width and direction of the histologic cut through the pulp of the maxillary first molar. Whenever possible the most central sections available were chosen for evaluation.

Establishing the ratio of labeled cells to the total cell population in the pulp was the major aim of this experiment. To accomplish this task it was necessary first to develop a simple counting procedure to determine the average number of cells per first molar pulp section in each of the seven age groups of rats.

The following is the technic employed for that purpose: one section through the maxillary first molar in each specimen (animal) of each age group was studied. All the pulp cells were counted in each of the six sections of each group, except the odontoblasts, the blood cells inside of the blood vessels and the endothelial cells. The cells were counted first in a high magnification (X 400) dry field of each of the six sections with the aid of an eyepiece grid reticular which divided the field in smaller areas making it easier to count the cells in the entire field. Then the number of high dry fields for each of the six pulp

sections was determined. In some areas the pulp was very narrow and it was necessary to count two half fields as one field. This was accomplished with a certain degree of accuracy with the aid of the reticular. The number of cells in the sections was then obtained by multiplying the number of fields in each section by the number of cells counted in one high dry field of it. The number of cells in all the specimens of each group were added and the total obtained was divided by six, thus obtaining an average number of cells per pulp of each age group.

Once the average cell population per pulp section was obtained for each age group, it was necessary to study the mitotic activity in the pulps at different ages by counting the cells which had incorporated the radioactive thymidine. This uptake indicated that these cells were in the process of desoxyribonucleic acid (DNA) synthesis preceding division, during the short period the isotope was available. In accomplishing the counts of labeled cells a series of steps were developed as follows:

The appearance of the labeled cells was studied. The nuclei of these cells appeared to be covered to various degrees by dark granules. These granules were photographic images on the NTB3 emulsion of radioactivity over and adjacent to the nuclei. There were also scanty background granules distributed throughout the entire slides.

In order to count the labeled nuclei it was necessary to establish first the background frequency of radioactive granules.

This was done by counting the number of granules in five squares of the eyepiece reticular in a high magnification (X 400) dry field, on the emulsion bordering the section of tissue. The number of granules thus obtained was divided by five and the resulting average was estimated as the background for that slide.

Although one square was large enough to contain more than one nucleus, it was decided to use one square area as a standard measure of the background square of the respective slide.

The counting procedure for the labeled cells followed a similar pattern to that described for the general counts. These counts, however, were made whenever possible, in nine sections of each animal of each age group and throughout the entire pulp of each section.

The labeled cells were counted in the following manner: Beginning at one corner of the section through the pulp, the slide was always moved from right to left in a set pattern of movements, never for more than one half of the field at any one time, thus studying the pulp tissue with consistent accuracy. The reticular served as a guide to avoid counting the same cell twice. After counting the cells within the series of fields in one row across the section, the slide was brought to the initial position and then moved upward one entire field and the right to left movement repeated for the adjacent row of fields. This was done successively until the entire pulp section was analyzed. Figure 1 illustrates the method schematically.

The total number of labeled nuclei counted in each section of each of the six specimens (animals) in each age group were added, and the resultant was divided by the number of sections. The average number of labeled cells per section thus obtained was divided by the average number of cells previously determined for the pulp sections of each group, and multiplied by one hundred. Thus a percentage of labeled cells per total cell population was obtained for each level.

Finally, an independent series of counts were made of the odontoblasts, in the 15 and 400 days old groups, in the following manner: Utilizing, whenever possible, the same region of the pulp chambers, five areas of the odontoblastic layer in one typical specimen of each age group were selected for counting the cells. The criteria for selection was that the sections at that area were the more centrally made.

The line of odontoblasts was positioned along the greatest diameter of the high dry field and the cells counted. Thus, counts were made of the odontoblasts along the same linear unit in the two age levels.

The purpose of these counts was to evaluate the difference, if any, between the numbers of these cells at a very young age (15 days) and at the oldest age studied (400 days).

CHAPTER IV

FINDINGS

1. General Morphology

At 10 days of age the maxillary first molar of the rat was incompletely developed and unerupted. The roots were unformed; however, the crown showed a complete outline form. The epithelial diaphragm was already present (Fig. 2) but the development of the root itself had not been initiated as yet. In outline, the pulp was large and highly cellular. The layer of dentin that surrounded this large pulp was relatively thin and by the same standard the predentin layer appeared almost as wide as the dentin. The connective tissue cells were evenly distributed throughout the pulp with the exception of the areas adjacent to the epithelial root sheath, and the floor of the pulp chamber, where a greater cell population was observed.

The odontoblasts were regularly arranged at the periphery of the pulp. Several sections showed areas in which there appeared to be a pseudostratification of the odontoblastic layers. Closer examination showed, however, that this appearance was due to tangential cut through laterally adjacent cells. The central sections presented a single row of columnar cells, tightly arranged in a "palisade" manner (Fig. 3) which contained a basophilic cytoplasm. These cells had basophilic oval-shaped nuclei

which stained darker than the cytoplasm but were not very dense.²⁵ Other cells observed were histiocytes, macrophages, extravasated erythrocytes, fibroblasts and a large main mass of immature stellate connective tissue cells. At the site of odontoblastic differentiation such cells appeared pear-shaped and or fusiform. Fibers were spread throughout the intercellular ground substance. Capillaries were observed evenly distributed throughout the entire pulp. Small loops extended even between the odontoblasts, and could be seen subodontoblastically next to the pre-dentin.

In the 15 days old specimens the formation of the roots was already initiated by a well developed epithelial root sheath. The tooth, however, was as yet unerupted. The coronal dentin appeared several times wider than the pre-dentin, but not so in the roots where the width of the dentin was approximately the same as that of the pre-dentin. The cell population and the rest of the pulpal contents remained similarly distributed as described at 10 days. In one specimen it was possible to observe a developing blood vessel of the caliber of an arteriole or venule entering the pulp in the center of the intermediate developing root (Fig. 4).

At 30 days of age the secondary dentin apposition was clearly demarcated by a basophilic line already visible in the pulp horns (Fig. 5). This basophilic line was an accentuation of the contour line of Owen that separated one calcified dentin layer from another. The secondary dentin seemed less tubular and showed

what appeared to be entrapped cells, probably odontoblasts. ²⁶ It was also lighter in color than the primary dentin. The layer of secondary dentin became increasingly wider in the older age groups. Between 30, 60, 90 and 125 days the pulp underwent few gross changes in appearance, except that the volume of the pulp continued to diminish.

At 400 days in the region of the pulp horns, the secondary dentin described a cone with the apex toward the pulp chamber. There seemed to be more odontoblasts per unit area than in younger specimens, covering the walls of the pulp chamber (Fig.6). The appearance then was that of a pseudostratified odontoblastic layer. These cells appeared more crowded even when there was a loss of some of them at 400 days. It appeared, also that the nuclei of these cells were smaller in size and more densely stained when compared with those of the youngest groups. By the same standard, the total cell size was apparently reduced, also, although the boundaries were not so clearly visible at 400 days of age. The cell population in the pulpal connective tissue was increased during the period of time between 125 and 400 days of age, as it can be observed in Table I. The average number of cells per section was even slightly higher than that seen at 30 days. The cells appeared closer together at 400 days in a diminished intercellular ground substance especially at the coronal pulp, where the reduction of the pulp chamber was more marked than in the roots. The outline of the pulp chambers be-

came increasingly irregular with increasing age. The collagen fibers were also evenly increased in thickness throughout the entire pulp. The nuclei of the connective tissue cells had approximately the same size and chromatin distribution at all ages studied. There were always nuclei which had several nucleoli, though seemingly these cells were less abundant as the animals were older.

Some cells that followed the course of the capillaries and were seen at 400 days of age for the first time in the alcian blue and periodic acid Schiff sections, showed characteristics of Mast cells; however, they were scanty and not clearly identifiable. Otherwise, the members of the cell population remained identical to that seen in younger specimens. The blood vessels increased in size and appeared in greater numbers at 30 days and remained without apparent changes at 400 days.

2. Study of the Fibers and Ground Substance with Special Stains

Silver Impregnation

The rat maxillary first molar pulp showed a predominance of fine reticular fibers, that stained black in color and appeared evenly distributed, in the 10 days old group. Collagenous fiber bundles as noted by the rose stain, were observed at the area of the dental follicle, some of them extended beyond the Hertwig's epithelial root sheath and technically they were considered within the pulp, but did not seem to originate there. The free ends of Hertwig's epithelial root sheath extended into the edges

of this band of rose staining collagenous fibers, which in the extraradicular position tended to cap over the end of the developing root. These bundles were not clearly evident throughout the remaining pulp. Korff's fibers were seen between the odontoblasts.

In the layer of loose connective tissue between the cap of collagenous fibers and the fundic bone there were numerous large patent blood vessels, which showed no evidence of impingement by forces related to eruption.

At 15 days of age, a slight increase of the collagenous fibers was observed, mainly in the developing roots, but the reticular fibers still had predominated in the pulp. The collagenous fibers increased in proportion to the reticular fibers, particularly in the root pulp, between 30 and 400 days. The Korff's fibers appeared denser than at 10 and 15 days and were readily seen after 30 days. As the animals increased in age, the pulp appeared progressively more fibrillar. The fibers also became coarser than in the younger specimens.

The predentin and dentin contained mature collagen fiber bundles as indicated by a rose stain.

Mallory's Connective Tissue Stain

At 10 days of age the rat molar pulp showed few blue staining collagenous fiber bundles at the areas of root development. Delicate fibers were abundant, however, and evenly distributed throughout the entire pulp (Fig. 7). Toward the proliferative

areas the collagen bundles seemed to be an extension of the connective tissue surrounding the region of root formation, which had the appearance of a sling that capped the developing root (Fig. 8). The same general pattern was seen in the 15 days old specimens. A slight increase in collagenous fibers was observed, however, at the developing roots and the floor of the pulp chamber. A gradual increase in collagenous fiber content was observed in the molar pulp between 30 and 400 days of age. This, again, was more evident in the root pulp and around the blood vessels than in the coronal pulp. At 400 days, however, the collagenous fibers were seen in thicker bundles in both, coronal and radicular pulps (Fig. 9).

The predentin and dentin contained high amounts of collagen in all the teeth examined.

Alcian Blue and Periodic Acid Schiff Stains

In the 10 days old specimens the pulp ground substance contained mainly acid mucopolysaccharides while neutral mucopolysaccharides also were seen. The odontoblasts cytoplasm were stained violet showing a content of acid mucopolysaccharides. Throughout the pulp, the cell boundaries of the young connective cells were not so clear. Only scanty fibers were observed coated with acid mucopolysaccharides, which appeared violet in color. At 15 days of age some specimens seemed to show a greater proportion of neutral than acid mucopolysaccharides in the ground substance, as seen by a magenta stain. After 30 days the ground

substance was mostly blue and the presence of acid mucopolysaccharide coated fibers in the roots and around the blood vessels was observed increasingly though never in great quantities. At 400 days of age the ground substance became increasingly more acid as seen by the density of the blue to violet color. The walls of the blood vessels throughout the pulp and at all the age groups studied contained acid mucopolysaccharides. The dentin was densely stained for acid mucopolysaccharides as seen by the violet stain in the most external layer and blue violet toward the pulp. The predentin was stained blue.

3. Autoradiography

The results of the autoradiographic study are expressed in Table I and Figure 10. The greatest frequency of labeled pulp cells in the rat molar occurred in the two younger age groups (10 and 15 days). Furthermore, the number of labeled cells increased from 92 to 127 between the tenth and fifteenth day. After 30 days the percentage of labeled cells was stabilized at a low but constant level throughout the period of the experiment. Yet at 30 days the labeled cells were concentrated in the growing roots. In the 60 days old group there was one specimen which showed four labeled cells in nine sections. This differed significantly from the other specimens in the group, which averaged 3.74 labeled cells per section. The 90 days old group presented the lowest percentage of labeled cells per pulp section. It is interesting to note that in the 125 days old group the average

number of labeled cells per section was increased over the two preceding groups. It should be mentioned however, that although five of the six specimens in the 125 days old group showed a similar, moderate number of labeling reactions, it was the sixth one which presented an unexpectedly high frequency of labeled cells, 95 labeled cells in nine sections. Nevertheless, it can be said that in general there is stability in the averages of labeled cells from 30 to 400 days of age.

Most of the labeling reactions in the 10 and 15 days old groups occurred in the areas of pulp proliferation (Figs. 11 and 12), that is where the roots were developing, while a considerable decrease in the frequency of labeled cells throughout the remaining pulp in the same specimens was observed (Fig. 13). In the older specimens with completely formed roots this reduction was markedly continued until a similar balanced pattern of cell division was reached, common to any further age period. The same observations were made for the radicular pulp as for the coronal pulp (Figs. 14 and 15).

Figure 16-I, II, and III are schematic representations of the distribution of labeled cells at 15, 30 and 400 days of age respectively.

CHAPTER V

DISCUSSION

The first pulp characteristic to be considered is the presence at all the ages studied of labeled cells, which is indicative of DNA synthesis, and therefore cell division. This suggests that the connective tissue cells of the pulp are constantly being renewed by mitotic activity, giving this tissue a balanced cell turnover and thus a permanent capacity for limited growth and repair. From this standpoint it may be considered that the molar pulp remains young in at least one aspect for at least 400 days.

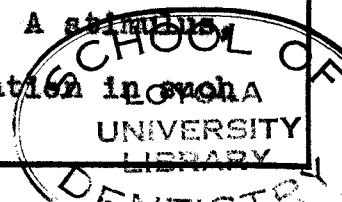
It is interesting to note, however, the remarkable proliferative activity in the pulp at 10 days of age and furthermore the increased cell proliferation through 15 days. During this period the roots begin to form and the pulp grows very rapidly. Some of the cells being produced are differentiated into odontoblasts, but most of them are integrated into the pulp tissue. This increase in pulp cells and the intercellular mucopolysaccharides they produce accounts for the development of the eruptive force that moves the tooth into the oral cavity. More of these cells are continuously produced as the roots grow, thus contributing to maintain the process of eruption during its early period.

The fact that the loose connective tissue and blood vessels beneath the sling of collagenous fibers and above the fundic

bone show no signs of impingement, particularly as evidenced by the patency of the veins, indicates, on a morphologic basis, that the biologic force resulting from cell proliferation and elaboration of intercellular substances is confined immediately adjacent to the open surface of the developing root. The force directed at the sling apparently is the most significant one participating in the active eruption of the rat molar.

The sling arrangement is histologically quite similar to, but not identical to, the hammock ligament described by Sicher (1942) for a continuously erupting tooth and for a single root human tooth.

Once the formation of the roots is completed and the tooth has erupted into the oral cavity, somewhere between 15 and 30 days of age, the proliferative activity is sharply reduced and even the cell population of the pulp is markedly diminished (Table I). Although the molar erupts throughout the life span of the rat long after the roots have formed, it can be attributed mainly to cementum apposition in the apices and in the crouch of the root stock, and bone apposition at the fundus and intraradicular crest of the socket. Having fulfilled its main function, the pulp if not disturbed remains in an essentially stationary state, as far as growth is concerned; at least certain cells always being renewed, but not accomplishing any defined function, beyond normal maintenance of the tooth. A stimulus, however, could increase the rate of cell proliferation in such



pulps. Also they may differentiate as well, as seen in those cases of pulp repair after being experimentally injured (Berman, 1957; Miyamoto, 1957; Kiryati, 1958 and others).

The pulp chamber reduces in size due to secondary dentin apposition and brings about an increase in the number of cells per unit area, as exemplified at 400 days of age when compared to the preceding four age groups. The rate of cell turnover at 400 days is only slightly lower than that of those four younger groups mentioned, except for the 90 days old group, however, the cell population is moderately increased at 400 days of age.

In comparing the findings in the present study with other similar studies reported in the literature it can be observed that the rat molar pulp presents a common pattern of growth with that of the hamster (Hoffman and Gillette, 1962). In both animals the cell multiplication is highly concentrated at the areas of root growth. Furthermore, it is confirmed that there are few labeled cells as reported by Messier and Leblond (1960) in the rat molar pulp after its formative growth period is terminated.

The pulp cells low level of labeling after root formation also presents some common features with other connective tissues in the rat, where only moderate numbers of labeled cells are observed (Messier and Leblond, 1960).

Differences in cell proliferation between animals in the same age group can be attributed to the different proliferative

potencies of connective tissues which are suggested by Leblond et al. (1959). These potencies, it is thought, can be influenced by local conditions bringing about an increase or diminution in the cell proliferation.

In general, changes in the rat molar pulp with advancing age do not appear to be as marked as they are in other connective tissues. The rat molar pulp preserves some young connective tissue qualities, for as long as 400 days of age in the normal animal, which imparts its individual characteristics.

The study of the general morphology of the rat molar pulp at various ages shows, however, that characteristic changes occur within this tissue with increasing age.

The reduction in size of the pulp chamber described by Hoffman and Schour (1938, 1940) and corroborated by Berman (1957) is also confirmed in the present study. Although, as previously mentioned, the cell population increases at 400 days, as well as the number of collagenous fibers of the pulp, the size of the nuclei do not show variations with aging. Considering the constant nucleo-cytoplasmic ratio, as observed by simple light microscopy, it is apparent that the loss in the pulp volume is in its intercellular content.

The deposition of secondary dentin apparent toward the pulp horns in the 30 days old animal is in near agreement with Hoffman and Schour (1940) who described the initiation of secondary dentin apposition at 35 to 40 days of age. This difference may

be a reflection of the different strains of rats studied, the abrasiveness of the diet leading to difference in the rate of attrition, or, for that matter, interpretation of the histologic specimens.

The cellular content of the pulp tissue at various ages did not vary ostensibly in the types of cells but in the number of them per unit area. The types of cells described in the findings section of this experiment coincide with those shown by Kiryati (1958).

The Mast cells, however, described in the 400 days old specimens, have not been reported previously. Indeed, Schour and Van Dyke (1934) could not find Mast cells in the pulp of the molar of rats aged 34 to 495 days. The fact must be considered that animals from different strains may also show different characteristics. Furthermore, they used hematoxylin and eosin stains, in which Mast cells are very difficult to observe.

The morphology and cytology of the odontoblasts as well as their location are typical of these cells in other mammalian species, and agree with the findings of Wade (1964). It is interesting, however, to note that even when the regular and tightly arranged odontoblastic layer of the very young specimens decreases in cells as the rat becomes older, the overlapping of several cell layers gives the impression of a numerical increase because of a pseudostratification (Figs. 3 and 6). This suggests that these cells are not renewed by differentiation of

mesenchymal cells and that they live for as long as the tooth³⁷ remains vital.

Once the optimal blood supply is acquired by the molar pulp, it remains stable regardless of the age. The entrance of the main blood vessel to the pulp through the central portion of the root shown by Kindlova (1963) can be readily seen in Figure 4 which is a section of the first molar at 15 days of age. Although the capillaries are widespread in the pulp, reaching the odontoblastic layer, as observed by Kiryati (1958), this study does not corroborate his statement that these capillaries are also present in the predentin.

The collagenous fibers are more numerous at the root pulp regions at all ages analyzed. No explanation is suggested and further studies are necessary to explain this observation.

Coinciding with observations of Zerlotti (1964), who found similarities between the aging processes of the human pulp and other connective tissues, it is possible to correlate some of the changes in the rat molar pulp to those findings derived from the analysis of animal and human connective tissues. Sobel and co-workers (1953, 1954, 1956) reached similar results to those reported in this study (an increase in collagen fiber content with increasing age) when analyzing connective tissues from different organs of various animals. Myers and Lang (1946) and Faber and Moller-Hou (1952) find the same trend in the connective tissue of the human aorta. There is always an increase in

collagen content of connective tissues with aging. (Figs. 7 and 9)

The fact that the fibers appear to be thicker in the pulp of older animals is in accordance with electron microscopic studies in connective tissue of the skin of the rat (Gross, 1950) and of the human (Banfield, 1955).

Despite the fact that the collagenous fiber content of the pulp increases slightly with age, it should be stressed that its relative collagen content is very low when compared to the dentin or periodontal ligament.

In the ground substance studied with alcian blue and periodic acid Schiff stains, the presence of acid and neutral mucopolysaccharides is not abundant. It must be considered, however, that the mucopolysaccharides of the pulp might have been washed out during the histologic preparations. It is evident that, when observable, the highly aggregated acid mucopolysaccharides, that show a blue to violet color, are more easily seen than the neutral, which appear pink. In fact, after 30 days little evidence of neutral mucopolysaccharides is seen. This suggests that the mucopolysaccharides of the ground substance of the pulp are produced mainly in the acid form, or become acidulated soon after having been released by the cells.

The odontoblasts show a violet cytoplasm indicating the high acid mucopolysaccharide production within these cells. The difference in color observed in the dentin, from violet at the outer layer, to blue violet at the more recently calcified layer,

to blue at the pre-dentin, is indicative of the proportion of acid mucopolysaccharides in these different areas. The oldest dentin has the highest content, which decreases toward the recently calcified dentin and is even less in the pre-dentin. It appears from these observations that the process of calcification can only proceed when the mucopolysaccharides become more acid.

It is clear that despite this and other studies, much research needs to be done to clarify the effects of aging on all the elements of connective tissue in general and pulp in particular. The rat molar pulp seems to be a good test object for the advancement of such research and radioactive thymidine is an appropriate indicator for growth.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Seventy male albino rats aged 10 to 400 days (Groups of 10, 15, 30, 60, 125 and 400 days old) were injected intraperitoneally with tritiated thymidine and sacrificed one to two hours after injection. The maxillary molar regions were fixed in 10% neutral formaline, decalcified in formic acid and later embedded in paraffin. Longitudinal sections were obtained through the maxillary molar pulps and stained by several methods: Mallory's connective tissue stain, silver impregnation, combined alcian blue and periodic acid Schiff stains, and hematoxylin and eosin stains. Autoradiographs were also prepared on the hematoxylin-eosin-stained slides. Counts were made of the total cell population and of those cells labeled by the uptake of the radioactive thymidine. The percentage of labeled cells in the cell populations at the different ages, was calculated.

As a result of a general histologic evaluation and the study of the special autoradiographic preparations, the following conclusions are presented:

1. The rat molar pulp shows DNA synthesis and therefore cell division at all ages up to 400 days.
2. The rat molar pulp has the capability of growth and

repair at least for a period of 400 days.

3. The growth of the pulp is greater in the younger age groups analyzed. This proliferation is most likely an important factor in the active movement of the tooth into the oral cavity. After 30 days, when the maxillary first molar is already present in the oral cavity, the pulp remains, apparently in a stationary state throughout the remainder of the 400 days span.

4. The size of the pulp chamber is greatly reduced as the rat ages, due to the apposition of secondary dentin. This corresponds with a slight relative increase in the cell population at 400 days of age over the 30, 60, 90 and 125 days old groups.

5. Reticular fibers in the pulp are abundant whereas collagenous fiber bundles are infrequently observed at all ages, with the exception of the 400 days old specimens which show a higher quantity of collagen than younger pulps.

6. The pulp shows evidence of acid and neutral mucopolysaccharides content at all the ages studied.

7. The normal mature dentin shows a high content of acid mucopolysaccharide. The predentin, however, an essentially uncalcified structure, shows a lesser acid mucopolysaccharides content.

8. The rat molar pulp is a reserve of loose connective tissue similar to other connective tissues, as characterized by

a continuous, apparently balanced, cell turnover.

9. The 400 days rat molar is not senile, in terms of cell population or potentiality for growth.

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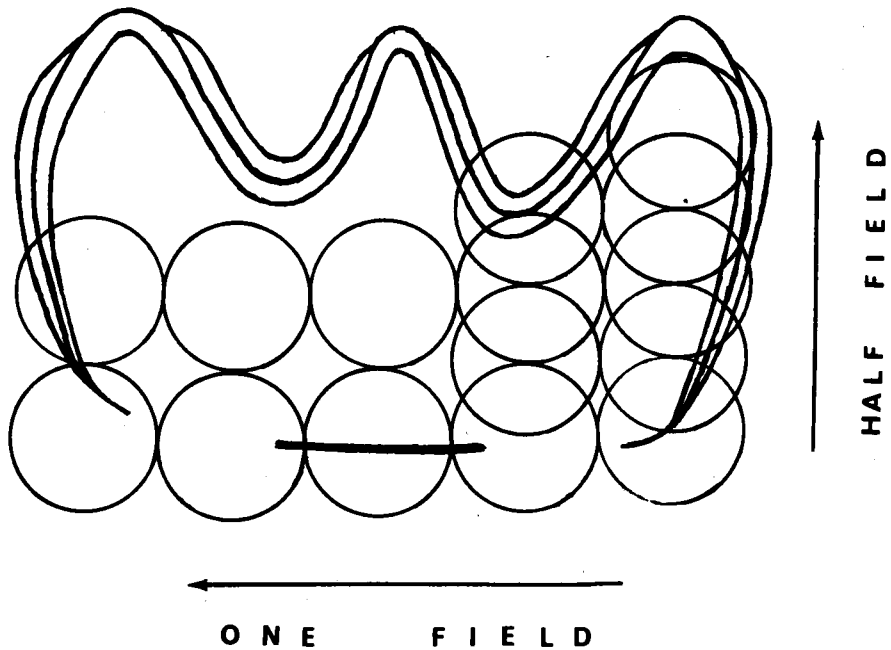


FIGURE 1. Diagram showing the direction of movement (arrows) of the slide, and approximate field size (circles), when counting the labeled cells in a 10 day old specimen.

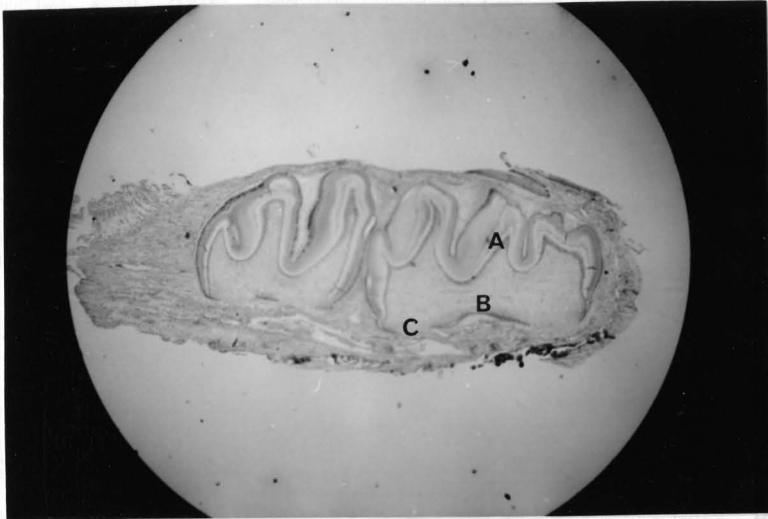


FIGURE 2. Photomicrograph of a longitudinal section of the maxillary molars at 10 days of age. A., first maxillary molar; B., epithelial diaphragm; C., area of root development. H & E Autoradiograph, (magnification X 2.5).

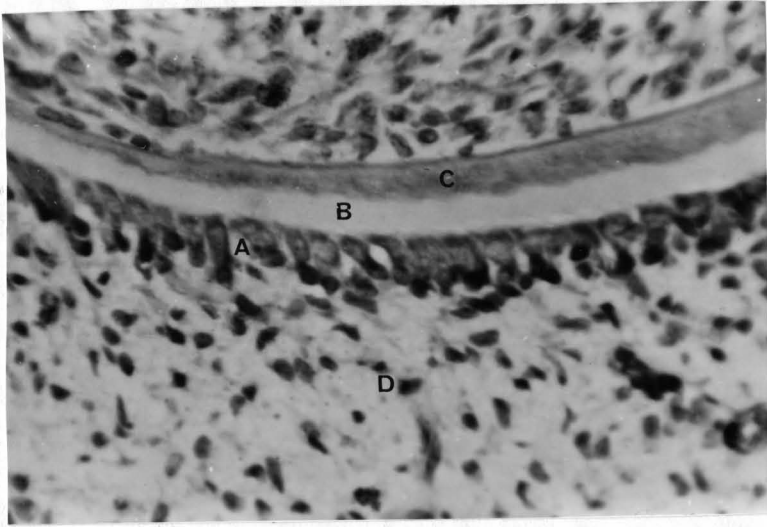


FIGURE 3. Photomicrograph showing odontoblastic layer at 15 days of age. The area shown is from the root near the coronal pulp. A., odontoblastic layer; B., pre-dentin; C., dentin; D., central pulp. H & E Autoradiograph (magnification X 100).

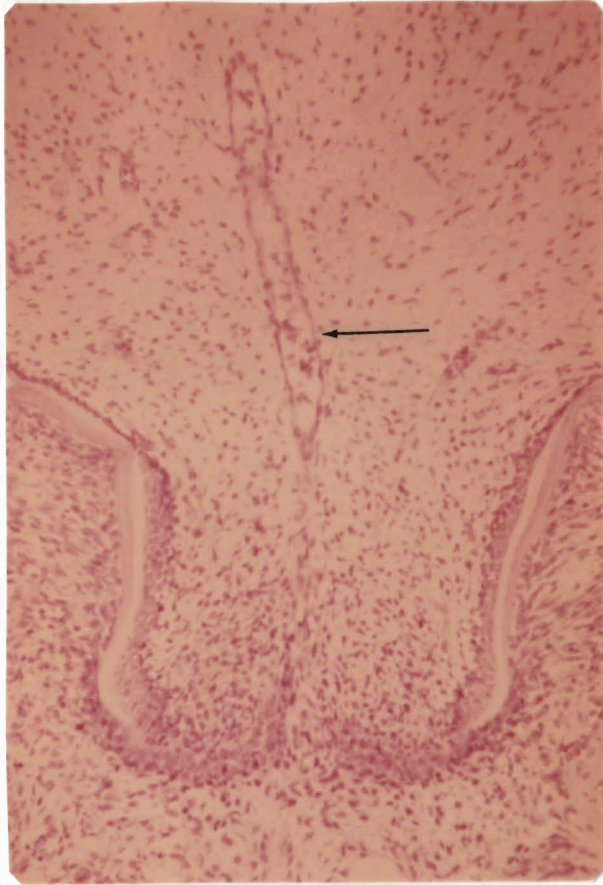


FIGURE 4. Photomicrograph of the medial root of a maxillary first molar showing a developing blood vessel entering the pulp (arrow) in a 15 day old specimen. H & E Autoradiograph (magnification X 72)

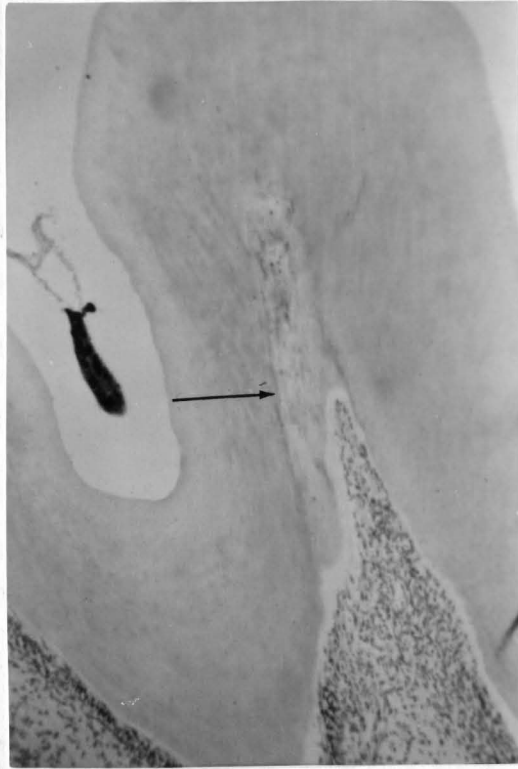


FIGURE 5. Photomicrograph illustrating the apposition of secondary dentin in a pulp horn of a 30 day specimen. Arrow illustrates the seam separating the secondary dentin from the primary. H & E Autoradiograph (magnification X 120).

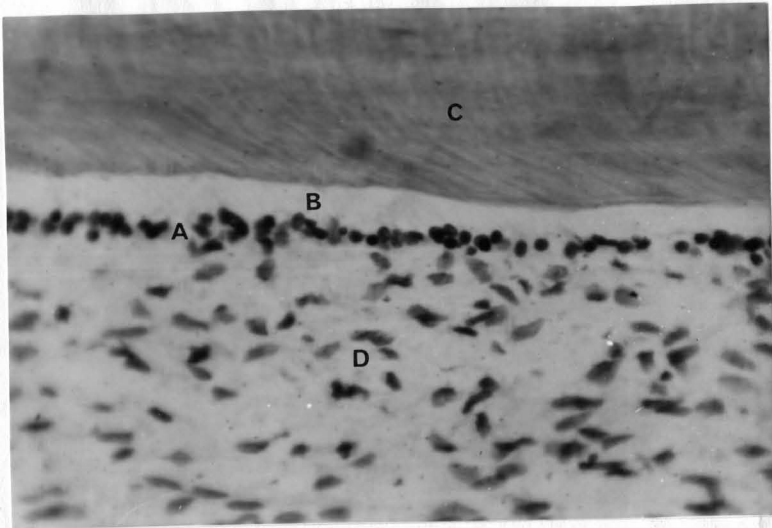


FIGURE 6. Photomicrograph of the odontoblastic layer at 400 days of age. The field is from the root near the coronal pulp. A., odontoblastic layer; B., predentin; C., dentin; D., central pulp. H & E Autoradiograph (magnification X 100).

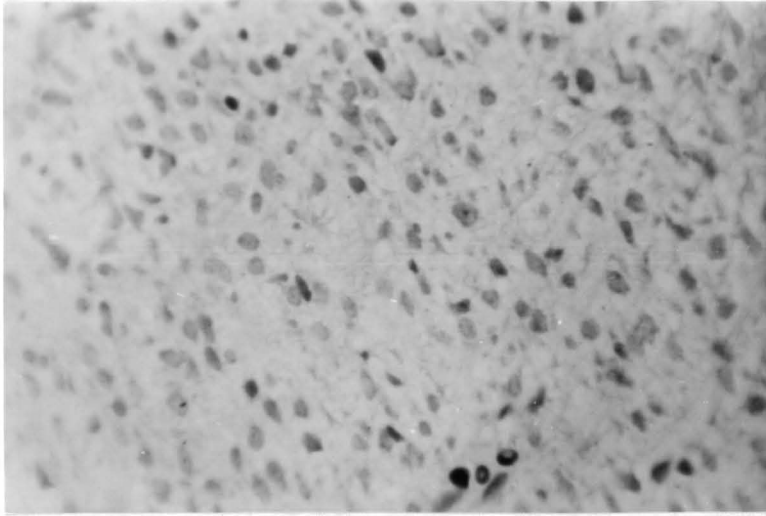


FIGURE 7. Mallory's connective tissue stain of a 10 day specimen in the area of a developing root. Only fine collagenous fibers are observed. (magnification X 100).

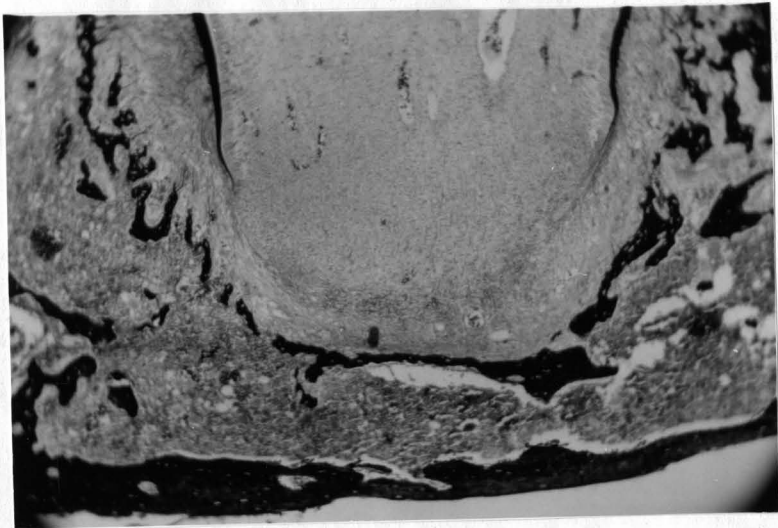


FIGURE 8. Photomicrograph showing the sling of relatively mature collagenous fibers capping the developing root. Blood vessels subtending the sling fail to show signs of impingement. Mallory's connective tissue stain (magnification X 25).

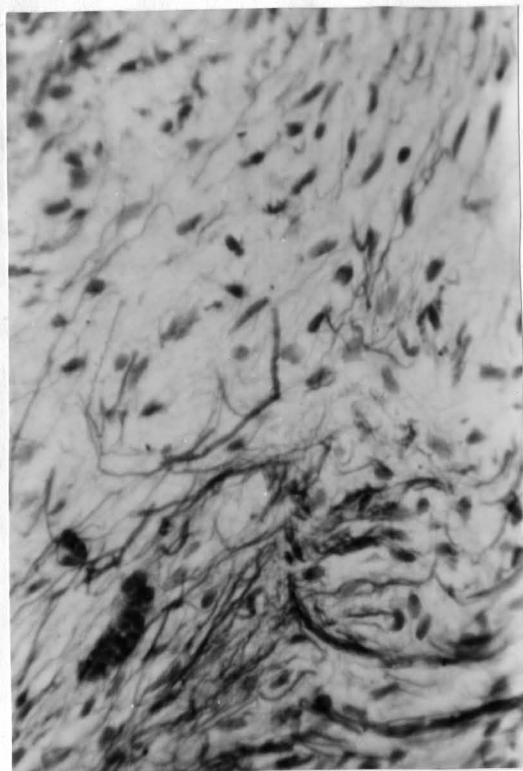


FIGURE 9. Mallory's connective tissue stain of a 400 day specimen in the region of junction of the radicular and coronal pulp. Heavy collagenous fiber bundles can be readily seen. (magnification X 100).

FIGURE 10. Graph showing the distribution of labeled cells at different ages. There is a sharp increase in labeled cells from 10 to 15 days of age, followed by an equally sharp decrease to 30 days, and showing a stabilized distribution between 30 and 400 days of age.

FREQUENCY DISTRIBUTION OF TRITIATED THYMIDINE
LABELED CELLS IN THE AGING RAT MOLAR PULP

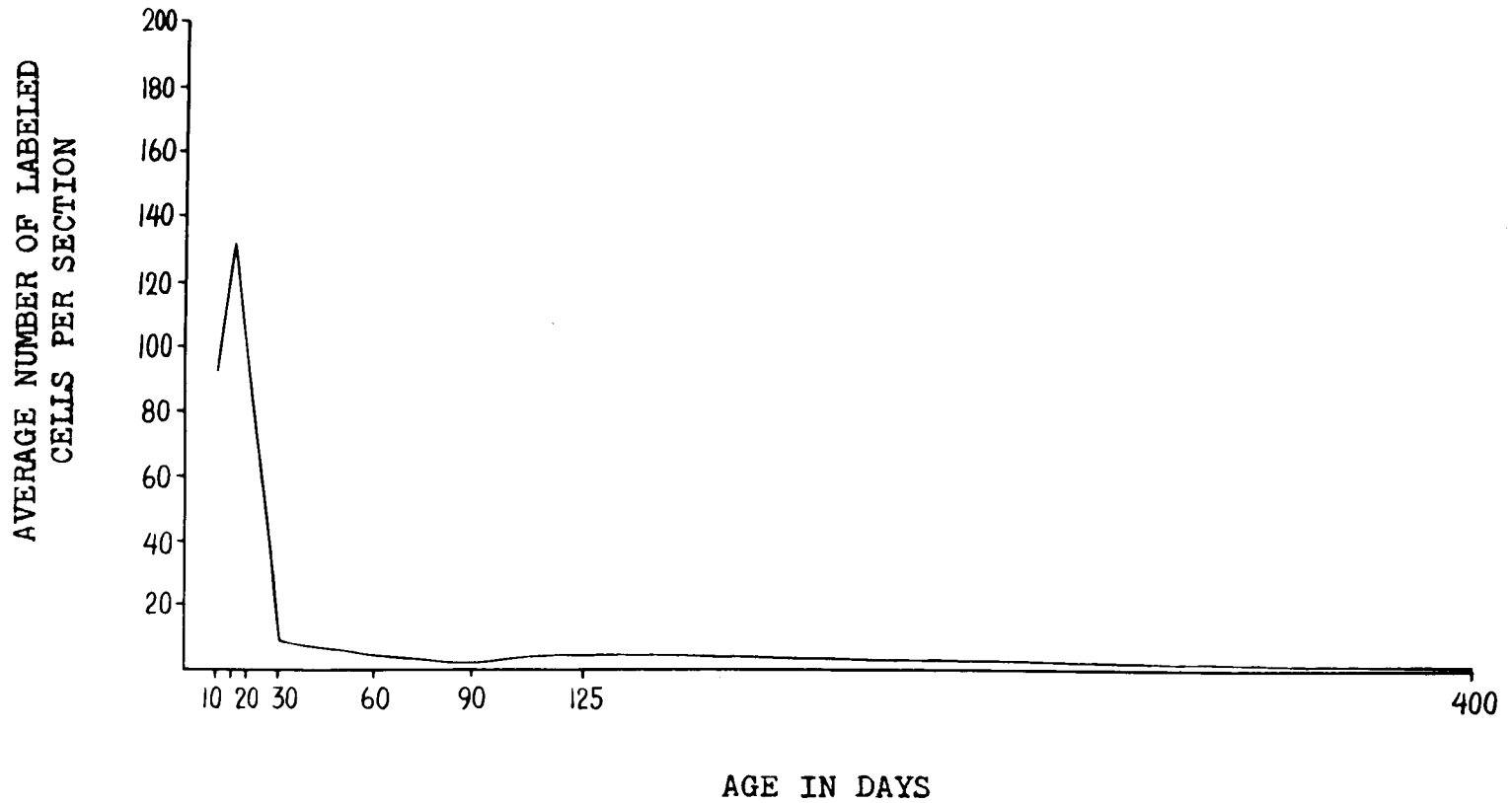


FIG. 10

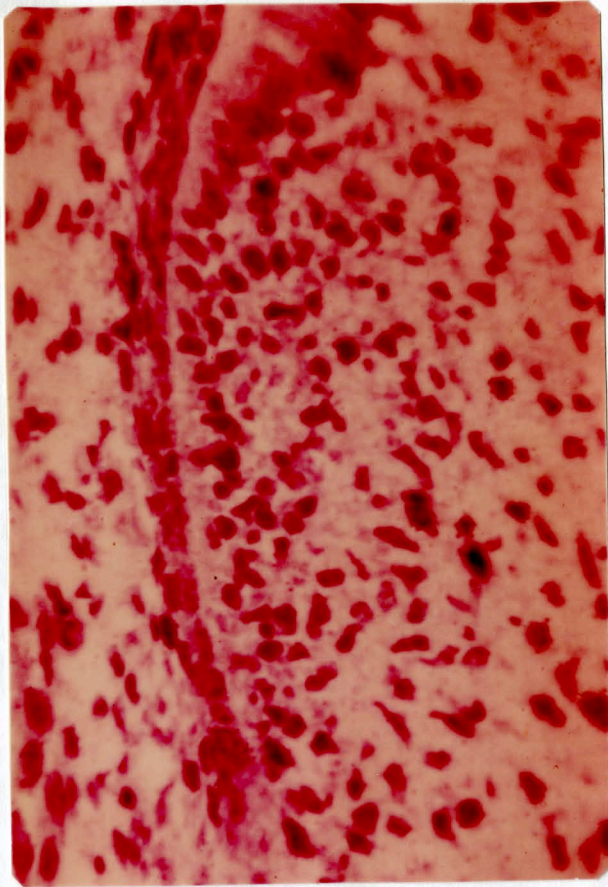


FIGURE 11. Photomicrograph of labeled pulp cells at 10 days of age at the area of the developing mesial root. H & E Autoradiograph (magnification X 540).

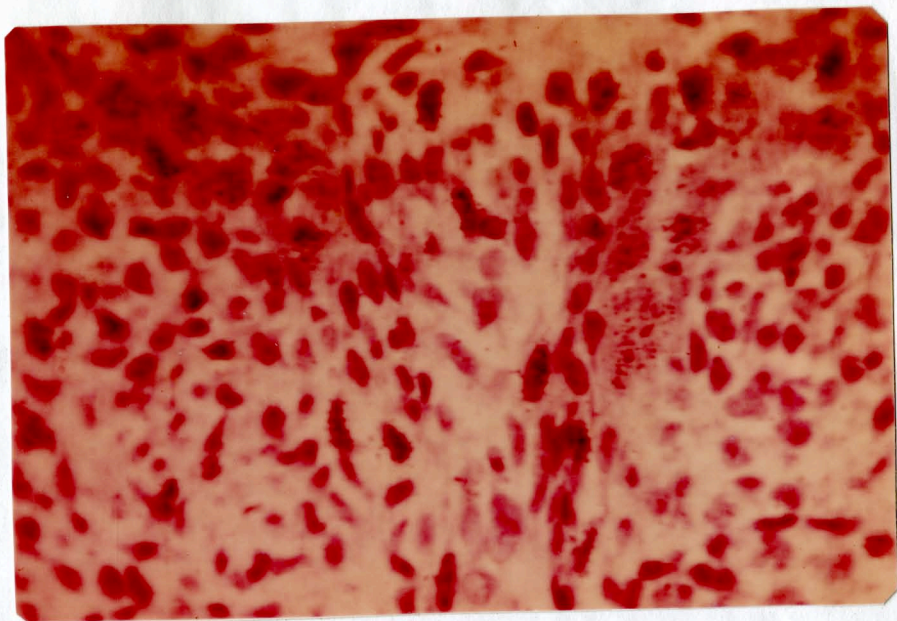


FIGURE 12. Photomicrograph of labeled pulp cells in a 15 day specimen at an intraradicular area. H & E Autoradiograph (magnification X 540).

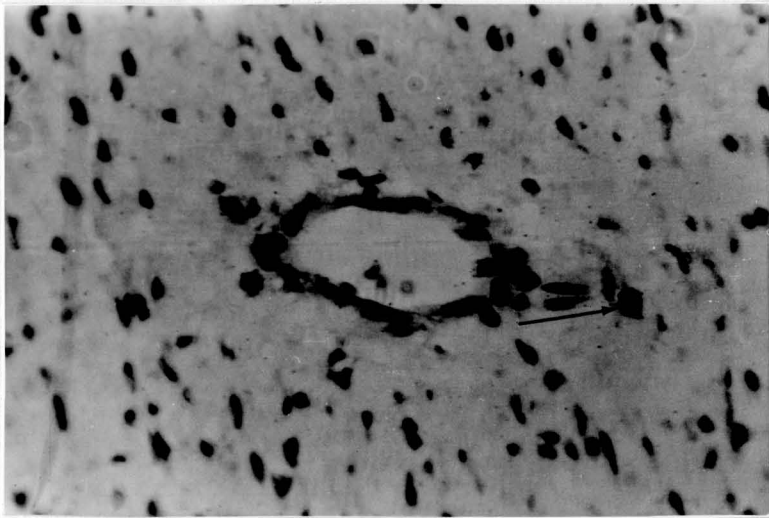


FIGURE 13. Photomicrograph of the central coronal pulp showing a developing capillary and an adjacent labeled perivascular pulp cell (arrow). Ten day maxillary first molar. H & E Autoradiograph (magnification X 450).

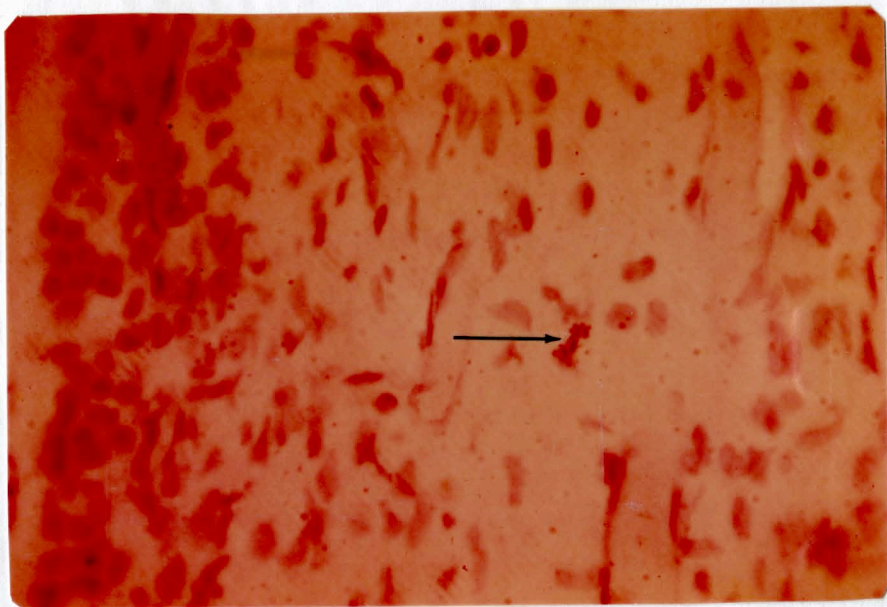


FIGURE 14. Photomicrograph of a labeled pulp cell at 60 days of age. Central root area (arrow). H & E Autoradiograph (magnification X 450).

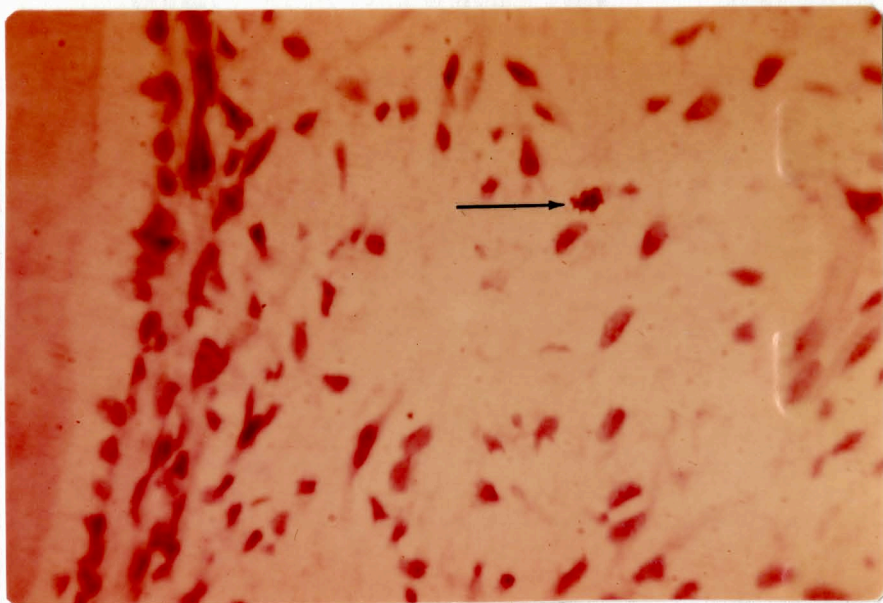


FIGURE 15. Photomicrograph of a labeled pulp cell at 125 days of age. Pulp horn (arrow). H & E Autoradiograph (magnification X 450).

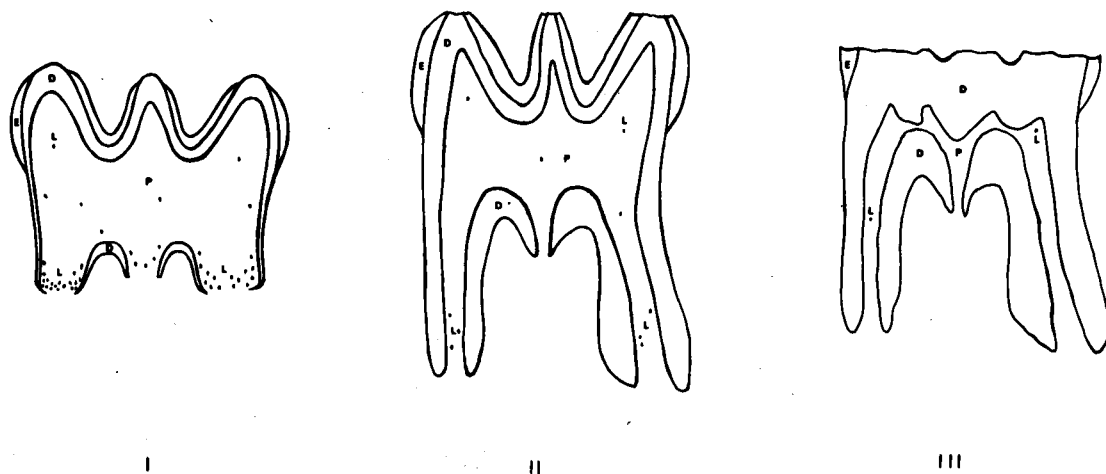


FIGURE 16-I, II, and III. Schematic representations of the distribution of labeled cells at 15, 30, and 400 days of age, respectively. E., enamel; D., dentin; P., pulp; and L., labeled cells.

FREQUENCY DISTRIBUTION OF TRITIATED THYMIDINE

LABELED CELLS IN THE AGING RAT MOLAR PULP

Age Groups	Average Number Of Cells Per Pulp Section	Number of Sections Per Animal For Counting Labeled Cells	Average Number of Labeled Cells Per Pulp Section	Percentage of Labeled Cells Per Pulp Sections
10 days	5793	52	92.44	1.60%
15 days	7412	51	127.72	1.72%
30 days	3470	49	9.34	0.27%
60 days	2509	52	3.17	0.13%
90 days	2404	50	0.86	0.04%
125 days	2761	54	4.44	0.16%
1400 days	3914	45	1.57	0.04%

TABLE I

APPROVAL SHEET

The thesis submitted by Dr. Rafael D. Pinzon has been read and approved by three members of the Graduate School Faculty.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 12, 1965
Date

Marvin Kizer
Signature of Advisor