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A Study of Certain Aspects of the Rat Molar Periodontium at Various Ages

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A STUDY OF CERTAIN ASPECTS OF THE RAT MOLAR PERIODONTIUM AT VARIOUS AGES

by

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I DEDICATE THIS THESIS TO

My Wife, Patricia,

and

My Mother and Father, Susie and Lee

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I. INTRODUCTION

The periodontium includes those tissue which invest and support the tooth, the gingiva, the cementum of the tooth, the periodontal ligament and the alveolar supporting bone.

The periodontium is subject to normal morphologic and physiologic functional variations with aging. According to Massler (1956) the various cells become less active, and the osteoblasts and fibroblasts are able to repair the wear and tear of daily function less rapidly and less completely with the result that as age advances the periodontium becomes atrophic.

It is known that aging tissue is less capable of initiating cell division and less capable of undergoing rapid differentiation (Sinex, 1966). It is also known that in aging connective tissue the gel to fiber ratio decreases (Sobel and co-workers, 1953, 1954, 1956 and 1958), the number of argyrophilic reticular fibers changes gradually into collagenous fibers (Gross, 1950) and that collagen fibers increase in width (Barnfield, 1955).

The purpose of this investigation was twofold: it was primarily intended to establish an index of connective tissue cell proliferation in the periodontal ligament of the upper first molar of the rat at various ages, and to determine the histologic appearance of the intercellular connective tissue of the periodontal ligament, the alveolar bone and cementum at various ages.

II. REVIEW

A. General Description of the Periodontal Ligament

The periodontal ligament is derived from the mesenchymal tissue of the dental follicle. From such tissues differentiate the chief components of the ligament: fibrocytes and collagen fibers bound together by a mucopolysaccharide ground substance and blood vessels. In addition, nerve fiber bundles and scattered cell rests form in the ligament.

Some standard texts in oral histology such as Sicher, (1962) and Schour, (1960) divide the collagen fiber bundles of the periodontal ligament of human teeth into three main groups as follows: gingival, transeptal and alveolar. The gingival group of fibers extends from the cervical cementum to the free and attached gingiva. The transeptal group extends from the cementum of one tooth to the cementum of the adjacent tooth. The alveolar group of fibers extends between the cementum and the alveolar bone. This group is divided into five subgroups. Alveolar fibers which extend from the cervical cementum to the crest of the alveolar bone are

called alveolar crest fibers. Fiber bundles which extend from the cementum to points directly across on the alveolar bone are known as the horizontal group. Fiber bundles which run from the cementum to points located coronally are known as the oblique group. The apical group is located in the apical area, these fiber bundles extend from the cementum directly across to the surrounding bone. The interradicular group of fibers run from the cementum at the furcation of multirooted teeth to the crest of the interradicular bone.

According to Sicher (1954) the fiber bundles run directly from bone to cementum, but individual fibers do not span the entire distance. The bundles are "spliced" together by shorter fibers in an intermediate plexus midway between cementum and bone. It is assumed that this plexus is common to the periodontal ligament of all mammalian teeth.

Bernick (1960) and Zwarych and Quigley (1965) were unable to find evidence of an intermediate plexus in the periodontal ligament of rat molars. They claimed to be able to trace individual fibers from cementum across the entire width of the ligament to the alveolar bone.

The fibers, cells, blood vessels and nerves of the periodontal ligament are embedded in a mucopolysaccharide ground substance.

Gersh and Catchpole (1960) classify the major constituents of the ground substance of connective tissue in the following manner:

- Probably of Local Origin
  - Acid Mucopolysaccharides
    - Hyaluronic Acid
    - Chondroitin Sulfate A
    - Chondroitin Sulfate C
    - Chondroitin
    - Chondroitin Sulfate B
    - Keratosulfate
    - Heparitin
  - Neutral Heteropolysaccharides
  - Protein
  - Soluble Collagen
  - Water
  - Enzymes
  - Immune Bodies
  - Metabolites
- Albumen
- Globulins
- Vitamins
- Hormones
- Ions

Originating from the plasma

These same authors believe that the ground substance of connective tissue is organized submicroscopically as a two phase system. The water-rich (less-dense) phase existing as submicroscopic vacuoles enclosed and separated from each other by the denser (colloid-rich phase). The phases are assumed to be in thermodynamic equilibrium.

According to standard texts on oral histology (Sicher, 1962, and Schour, 1960) most cells of the periodontal ligament are typical fibroblasts. They lie at the surface of the fiber bundles and are probably active in the formation and maintenance of the principal fibers. Cementoblasts are found on the surface of the cementum; these cells are present throughout the life of the tooth and are the source of cementum. Osteoblasts lie on the alveolar surface of the periodontal ligament. They line those areas of the socket wall on which bone apposition occurs. Multinucleated osteoclasts are present on the wall of the bony socket in areas which are undergoing bone resorption. Epithelial rests of Malassez are found scattered throughout the ligament. They are thought to be remnants of the epithelial sheath of Hertwig.

The blood supply of the periodontal ligament is derived primarily from the interdental and interradicular arteries.

Other sources of blood supply to the ligament are the apical and gingival arteries.

Generally the nerves of the ligament follow the path of the blood vessels.

B. The Rat Molar and Periodontium

According to Schour and Massler (1949) the histology and physiology of the dental tissues of the rat molar are quite similar to those of human molars; in particular, the periodontal tissues approximate closely the human periodontium.

According to O'Brien, Bhaskar and Brodie (1958) the developing first molar tooth of the rat is initiated at 13 days insemination age, the inner enamel epithelium differentiates into ameloblasts and adjacent connective tissue cells form odontoblasts with subsequent dentine formation at 21 days insemination age. At 3 days after birth, enamel formation begins. At 10 days after birth, the formation of the root begins and Hertwigs epithelial sheath appears. The tooth breaks through the oral epithelium around 17 days after birth and comes into functional occlusion at 23 days. Roots are completed at 30 days after birth.

Schour and Massler (1949) state that "secondary cementum in the first molar of the rat begins to form the 35th day and is concomitant with the functional stresses imposed upon the teeth and is thus added continuously throughout the life of the animal, so that in the adult rat one-third or more of the relatively long root is formed by cementum alone."

According to O'Brien, Bhaskar and Brodie (1958) in the early stages of development of the first molar tooth of the rat, the dental follicle consists of loosely arranged mesenchymal cells with abundant intercellular fluid. With age the follicle changes to a highly cellular structure with fusiform cells arranged parallel to the tooth surface. Later, collagen fibers appear among the fusiform cells. When the tooth erupts into the oral cavity, further organization of the periodontal fibers occur, and they extend from the bone to the cementum. When the tooth comes into functional occlusion, the periodontal ligament becomes densely collagenized and the periodontal space widens.

Sicher and Weinmann (1944) described the physiologic distal drift of the rat molar teeth in correlation with the growth of the maxilla and mandible. They found that apposition of bone along the mesial alveolar wall and resorption of bone along the distal wall were characteristic for all molar teeth.

C. Aging in Connective Tissue

Various changes in the structure and function of connective tissue have been demonstrated in advancing age.

Sobel and co-workers (1953, 1954, 1956 and 1958) studied the ratio of collagen to hexosamine, in general, they found that the gel to fiber ratio decreases with aging in the skin of the rat, guinea pig, squab, rabbit and man, in the femur of the rat, and in the lung of the rabbit.

Clansen (1962a and 1962b) determined the age variations in the extracellular substance of the thoracic aorta, myocardium and skin of 35 normal human fetuses aged 11 to 19 weeks. He also studied the same tissues in autopsy material of 87 persons aged 4 months to 86 years who met with sudden death. In all three organs the connective tissue appeared normal on gross examination, but Clausen could demonstrate a significant and steady decrease in the hexosamine to hydroxyproline ratios with advancing age. These results indicate a steady increase in the fibrous components in relation to ground substance.

The predominance of argyrophilic reticular fibers in the skin of the newborn rat which changed gradually with aging into collagenous fibers was discussed by Gross (1950).

Barnfield (1955) studied abdominal skin samples from three fetuses measuring 2.5 cm. to 18 cm., a fetus seven months of age, a child of 3 years, and three adults aged 65, 68 and 85 years. In general, he found that collagen fibrils increased in width with advancing age.

Asboe-Hansen (1963), after analyzing the findings of several authors, concluded that the fibrillar density is increased as the connective tissue becomes older.

Ring (1960) studied histologic and histochemical age changes in oral subepithelial connective tissue in Sprague-Dawley rats. He found that histologically identifiable age changes began to appear in oral and dermal connective tissue at about one year of age, but did not become marked until about 500-600 days. Later changes included loss of discreteness of collagenous fibers and homogenization of intercellular areas generally. Histochemically basement membrane and endothelium showed an increase in thickness with aging. Periodic acid-Schiff background staining of connective tissue appeared to increase to a maximum at 500 to 600 days and then decrease. Alcian blue had its greatest intensity at 500 to 600 days. The hexosomine to collagen ratios of rat oral mucous membrane declined throughout life.

Gersh and Catchpole (1960) suggested that with increasing age there is a change in the nature of the colloidal aggregates of ground substance of connective tissue, including that of basement membranes. This consists of a relative increase in the denser phase and a relative decrease in the water-rich phase of the ground substance. Whether this influences the process of aging of cells is not known.

D. Age Changes in the Rat Periodontium

A review of the literature revealed very few studies on the effects of age on the periodontal structures in experimental animals.

Hoffman and Schour (1940) described the appositional patterns of growth of alveolar bone and cementum using alizarin-injected rats from 14 to 500 days of age. They concluded that apposition of alveolar bone of the crests and fundi and of secondary cementum was a continuous process throughout the life of the animal.

Sherpo (1947) studying Wister albino and Norwegian gray rats aged 21 to 1,170 days, described a physiologic atrophy of the interdental septum. The incidence ranged from zero at 21 days to 90% at the 970 to 1,170 day old group.

Belting (1952) studied the periodontium of albino and Norwegian gray rats aged 21 to 1,000 days. He found that the distance between the alveolar crest and cementoenamel junction increased with age. The rate of increase in distance between these two points diminished as age advanced. Bone apposition occurred at the alveolar crest between 21 and 900 days of age. Bone resorption was occasionally seen at the alveolar crest in the very aged animals. A decrease in width of the interdental septum with age was described. Root resorption occurred most commonly on approximating root surfaces of adjacent teeth, and the number of areas of resorption tended to increase as the age of the animal increased.

Bernick (1960) studied age changes in the blood supply to the molar teeth of rats aged one to 18 months. Blood vessels were demonstrated by the saline-India ink-gelatin perfusion method. The vascular supply to the periodontal ligament of young animals was found to be similar to the standard textbook descriptions. On the other hand, with age there was a progressive thickening of the bony trabeculae resulting ultimately in fused dens bone. As a result of this osteosclerosis there was a gradual loss in the number of perforating vascular branches. By the time the rat reached the age of one year there was a complete absence of demonstrable perforating branches.

E. Autoradiography

Ionizing radiations act upon the photographic emulsion in the same manner as light does. If a piece of film is exposed to an object containing radioactive material, a photographic image is produced upon development, which provides visualization of the location of the radioactivity in the sample. This image is known by such names as autoradiograph, autoradiogram, autogram and radioautogram.

According to Comar (1955) the great importance of autoradiography from the biologic standpoint is the fact that the technique permits study of cellular function at the cell level. It becomes possible to develop correlations between cytologic structure, cellular physiology and pathology, physiochemical properties of the cells and the location pattern of specific chemical elements introduced into the system.

Various experimental studies have demonstrated that tritium labeled thymidine (Thymidine-H^3) is a specific precursor of desoxyribonucleic acid (DNA) (Reichard and Estburn, 1951, Fitzgerald, 1955, Lajtha and Oliver, 1951). Hughes (1958) stated that studies on DNA synthesis show no evidence for turnover of DNA. He believes that a cell synthesizing DNA is a cell preparing to divide. Exceptions to this include polyploidy and polyteny in which cell

division does not follow DNA synthesis.

DNA labeling experiments by Hughes et al (1958), Walker (1958) and Cronkite (1959) have shown that the synthesis of DNA occurs late in interphase with a long presynthetic period following telophase and a shorter postsynthetic period preceding prophase. During this period of DNA synthesis, thymidine is incorporated into new DNA.

In order to label and identify cells which are synthesizing DNA, thymidine containing tritium, a radioactive substance is used. The physical properties of tritium and its low energy beta electron application to autoradiography have been discussed by Gross et al (1951), Hughes et al (1958), Hamilton (1959), Lajtha and Oliver (1959) and Cronkite et al (1959).

As discussed by Hoffman (1962) tritium is an isotope of hydrogen having a mass of three. The beta particle emitted during this decay is one of the least energetic known. Maximum range of the particle is said to be 6-8 microns in water and tissue and only 2 microns in photographic emulsion. This short range particle accounts for good high resolution autoradiographs since all of the silver grains activated by the particle will lie in clusters within one micron of the labeled nucleus. However, the overall efficiency of tritium

is low, of the order of 2% in tissue specimens and 5% in smears. This is because of the short range of the particle and also because less than 50% of the particles travel initially in the direction of the emulsion.

Labeling of new DNA by injected tritiated thymidine is almost instantaneous. Quastler and Sherman (1959) found intestinal crypt cells of mice had half their final amount of isotope within five minutes after injection of thymidine-H^3. Saturation was reached in approximately 10 to 20 minutes. Lajtha and Oliver (1959) reported the plasma clearance is probably less than ten minutes. In dogs, 99.9% of the injected dose disappears from the plasma in five minutes.

According to Cronkite (1959) emulsion sensitivity is such that dosages of 0.1 to 0.2 microcuries per gram of body weight with exposures of 30 to 60 days can produce very adequate radioautographs. This low dosage is desirable since radiation damage within the nucleus is the severest limitation of the use of triated thymidine.

F. Studies on Cell Proliferation

1. Connective Tissue Proper

Schultz and Oehlert (1959) and Leblond, Messier and

Kipriwa (1959) studying the uptake of radioactive material in several tissues of the mouse and rat found what they described as "surprisingly" high numbers of labeled connective tissue cells in these tissues.

Messier and Leblond (1960) studied cell proliferation and migration after injecting thymidine-H^3 into mice and rats. The animals were sacrificed from one hour to 95 days after injection. Most connective tissue cells exhibited a moderate frequency of labeling, being 0.2% to 1.0% in the derma. Labeled nuclei were found in the pulp of the molar teeth, and in the periodontal ligament of the continuously growing incisor teeth.

2. Periodontal Tissues of Teeth of Limited Growth

Muhlemann, Zander and Halberg (1954) studied mitotic activity in the periodontal tissues of male black rats five months of age. The lower jaw was sectioned in a mesiodistal direction, and the periodontal ligament adjacent to the roots of the three molars was referred to as a "periodontal membrane unit." The number of mitoses in three periodontal membrane units per rat was recorded, the number of resting cells was not recorded. On the average 23 mitoses were found per 3 periodontal membrane units. Thirty-three percent of all mitoses were found in the periodontal ligament of the

first molar, 30% in that of the second molar, and 37% in the periodontal ligament of the third molar. The majority of the mitoses recorded were from fibroblasts (69%); 31% were endothelial cells.

The above authors observed mitoses throughout the whole width of the periodontal ligament, however, mitotic figures were more frequent in the neighborhood of the bone. Considerably more mitoses were recorded for the regions adjacent to the furcation and around the apices of the roots than in the rest of the ligament. Small accumulation of mitoses were recorded around the coronal part of the acellular cementum except for the distal side of the mesial roots where no mitoses were found.

Hoffman and Gillette (1964) utilizing radioautography studied mitotic patterns in the developing molar roots of hamsters aged 5 to 30 days. In the periodontal ligament the greatest frequency of labeling was found just lateral to the root apex. The frequency of labeling was highest in the 4 to 7 day old group and gradually declined from high to low in the 7-13 day old specimens and was lowest in the 13 to 30 day old specimens.

Diab and Stallard (1965) in a study of the relationship between the epithelial root sheath and molar root development

in rats varying in age from birth to 35 days compared the distribution of labeled connective tissue cells between different areas within the ligament. During tooth eruption the number of cells undergoing DNA synthesis varied markedly between the apical and furcation areas. This variation was brought about by the small number of labeled connective tissue cells near the bony surface at the apex of all developmental stages when compared to the large number of labeled connective tissue cells next to the bone in the furcation. The reverse was true regarding labeled connective tissue cells in relationship to the cementum, with the greatest number present in the apical area.

McHugh and Zander (1965) studied DNA synthesis in cells of the periodontium of developing and erupted teeth in four Rhesus monkeys. Relative to the periodontal ligament, they found that several different types of connective tissue cells were labeled, most were spindle shaped fibroblasts or endothelial cells in vessel walls. Plump undifferentiated mesenchymal cells, osteoblasts and cementoblasts were also labeled.

Around erupting teeth, labeled cells were evenly distributed throughout the middle one-third of the periodontal ligament and the third next to the bone. The one-third

nearest the cementum contained very few labeled cells.

A similar distribution of labeled cells was found around teeth in functional occlusion, although the third of the ligament next to bone was less heavily labeled than the middle third.

When the distribution of labeling was related to specific areas of the ligament, no particular pattern could be detected except that during eruption there was significantly more labeling in the furcation area between the molar roots and very little around the developing apex.

III. MATERIALS AND METHODS

Maxillae from fifteen apparently normally healthy male hooded rats* aged 10, 15, 30, 60, 125 and 400 days were obtained for the present study.

The animals were grouped according to age in the following manner: the 10, 15 and 30 day old groups contained three animals per group; the 60, 125 and 400 day old groups contained two animals per group.

Each animal was injected intraperitoneally with tritiated thymidine**, specific activity 1.90 curies per millimole, at a dosage level of 0.7 microcuries per gram of body weight. All animals were anesthetized with ether and sacrificed within two hours of the intraperitoneal injection. Immediately following the sacrifice, the maxillae were dissected and then fixed in ten percent neutral formalin.

The molar region of the right maxilla from each animal was decalcified in formic acid***, dehydrated in ascending

*supplied by Abrams Stock Breeders, Chicago, Illinois
**supplied by Schwartz BioResearch, Inc., Orangebury, N. Y.
***Manual of Histologic and Special Staining Technics, published by the Armed Forces Institute of Pathology, Second Edition, Page 4, 1949.

orders of alcohol (75%, 95% and 100%) and embedded in paraffin. Histologic sections were cut from four to six microns and mounted with albumen on standard 3 x 1 inch glass slides.

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

Autoradiographs were developed on the same slides used for the hematoxylin and eosin stains following basically the technique used at the Medical Research Center, Brookhaven National Laboratory, Upton, New York, and compiled by Emil R. Adamik and published by Schwartz BioResearch, Inc., Orangebury, N. Y.

Since the radioautographic emulsion NTB_3* required refrigeration storage it was necessary to allow the bottle of

*Distributed by William A. Sykes, Research Division Special Products, Rochester, New York.

emulsion to come to room temperature. The emulsion was then placed in a water bath at 43°C for approximately fifteen minutes to change it from a gel to a liquid state. Approximately 40 milliliters of the liquid emulsion 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.

Slides were placed on a metal warming plate to warm at 43°C; they were then dipped three at a time into the Coplin jar containing the emulsion for a period of approximately ten seconds. The slides were then removed from the emulsion, drained on a paper towel and left standing in a drying rack with the frosted ends up, permitting the excess emulsion to drain. The emulsion was then permitted to gel on the slides for five to ten minutes before being dried with an electric nonheating fan. When dry the slides were stored in black boxes containing perforated capsules fitted with "Dririte" (a drying agent) and the boxes were sealed with black phstic tape. They were then kept in a refrigerator for two weeks.

The autoradiographic emulsion on each slide was developed in a dark room using a Wratten #4 safelight at a distance of four feet. The slides were developed for two

minutes in Kodak D-19 developer, rinsed for five to ten seconds, fixed in Kodak fixer for two minutes, then washed for ten minutes after which they were dried.

Histologic sections exhibiting the best central sections through the crown and two buccal roots of the maxillary first molar were selected for detailed study. The dental follicle and periodontal ligament were divided into a number of separate areas for radioautographic analysis. These areas were given letter designations as follows:

1. MAF - the mesial apical follicular connective tissue lying between the mesial epithelial root sheath and the underlying bone.
2. HF - the horizontal follicular connective tissue located between the epithelial diaphragm and the underlying bone in the furcation area.
3. DAF - the distal apical follicular connective tissue lying between the distal epithelial root sheath the underlying bone.
4. MMR - the periodontal ligament on the mesial surface of the mesial root from the cemento-enamel junction to the midportion of the apex of the mesial root.
5. DMR - the periodontal ligament on the distal surface

of the mesial root from the interradicular periodontal ligament to the midportion of the apex of the mesial root.

6. I - the interradicular periodontal ligament at the furcation.

7. MDR - the periodontal ligament on the mesial surface of the distal root from the interradicular periodontal ligament to the midportion of the apex of the distal root.

8. DDR - the periodontal ligament on the distal surface of the distal root from the cemento-enamel junction to the midportion of the apex of the distal root.

With the aid of a Whipple disk inserted into the eyepiece of the microscope at a magnification of 400X, the total number of connective tissue cells with the exception of endothelial cells and blood cells, were counted in each of the areas of the periodontal ligament studied, in each animal of each group. The number of connective tissue cells undergoing DNA synthesis in these same areas were then subsequently counted. The number of labeled connective tissue cells was then divided by the total number of connective tissue cells and multiplied by one-hundred thus obtaining a premitotic index for each area of the dental follicle and periodontal ligament studied. The

percentage of connective tissue cells undergoing DNA synthesis in each area of the periodontal ligament studied in each animal within a given group was averaged and presented as the average premitotic index per group in each of the seven groups of animals which were studied.

Before a cell was considered to be labeled, the following criteria had to be fulfilled:

1. Grains in the emulsion were required to be clustered over a nucleus.

2. Five or more grains over a given nucleus were required (background in a similar area was less than one grain).

IV. FINDINGS

A. General Morphology

1. Period of Root Formation

At ten days of age the maxillary first molar was completely encased in a bony crypt; however, the bone covering the occlusal surface of the developing tooth was only a few microns in thickness and was undergoing osteoclastic resorption.

The crown of the maxillary first molar at ten days of age exhibited a complete outline form. The outer and inner enamel epithelium had formed an epithelial root sheath consisting of a vertical segment and a diaphragmatic portion. A short segment of dentin had extended apically from the future cemento-enamel junction.

That portion of the dental follicle located between the epithelial root sheath and the bony crypt consisted of a loose connective tissue framework which was highly vascularized.

Using silver impregnation it was observed that the connective tissue contained a fiber content which was almost exclusively reticular in nature; few rose colored collagen fibers were present in this area. A relative scarcity of blue staining fibers was observed using Mallory's connective tissue stain.

Alcian Blue Periodic Acid-Shiff (AB-PAS) stain was used to study the connective tissue ground substance. In the 10 day specimens the ground substance of the connective tissue located between the epithelial diaphragm and bony crypt contained both acid and neutral mucopolysaccharides. The pink staining neutral polysaccharides were more abundant in this area than in the adjacent pulp tissue.

At 15 days of age the reduced enamel epithelium was apparently beginning to fuse with the oral epithelium. Root formation was now well underway. The mesial surface of the mesial root and the distal surface of the distal root appeared to have reached approximately one-fourth of the future length of the dentinal portion of the root. A well formed furcation was also present at this age. The periodontal ligament appeared to be wider in the teeth of the 15 day old group than in any other age group studied, and was highly cellular and vascular. In this age group the periodontal ligament could be divided into three cellular layers;

a layer of cementoblasts lining the root surface, a layer of osteoblasts lining the surface of the alveolar bone, and a wide, intermediate layer of fibroblasts and mesenchymal cells.

The periodontal ligament in this age group contained only very few, scattered, black staining reticular fibers as seen with silver impregnation. The vast majority of fibers in the ligament as seen with both silver impregnation and Mallory's connective tissue stain were apparently fine collagenous fibers. Some of these collagenous fibers were attached to cementum, some were attached to bone, and a third group was found to be intermediate connecting the other two groups. In no instance could fibers be traced through the entire width of the ligament.

The connective tissue between the epithelial diaphragm and the bony crypt was similar to that described in the 10 day specimens.

New bone was being laid down principally in the furcation area and along the alveolar bone opposite the mesial surface of the mesial root.

When stained with AB-PAS, the cementum of the 15 day specimens exhibited a blue staining surface indicating the presence of acid mucopolysaccharides. Similarly, the alveolar

bone opposite the mesial surface of the mesial root and the interradicular bone both exhibited a moderately wide blue staining surface layer. The surface of the bone of the fundus exhibited a similar but thinner blue staining layer.

Both acid and neutral mucopolysaccharides were observed coating the fibers of the periodontal ligament, however, the acid mucopolysaccharides seemed to predominate as noted by the violet staining reaction with AB-PAS. In both the 10 and 15 day specimens, the walls of the blood vessels contained a predominance of pink staining neutral mucopolysaccharides.

2. Period of Functional Occlusion

At 30 days of age the maxillary first molar was in functional occlusion. The dentinal portion of the root was completed and a small amount of cellular cementum was present around the apical portion of the roots. The fibroblasts in the periodontal ligament were uniform in size and shape, and the nuclei were large and ovoid. The long axis of the fibroblasts were oriented horizontally in the periodontal ligament in the alveolar crest area and in the area of the furcation. In the remaining portion of the ligament the fibroblasts were oriented obliquely to the vertical root surface. The ligament contained three cellular zones as described in the 15 day specimens.

Areas of bone apposition and resorption were evidenced on the alveolar bone, but the majority of resorptive activity tended to be present in alveolar bone opposite the distal root surfaces, and the majority of bone apposition was seen on the alveolar bone opposite the mesial root surfaces and on the interradicular bone.

With the use of silver stain and Mallory's connective tissue stain, gingival, transeptal, and alveolar fibers could be identified. The alveolar fibers could be further subdivided into alveolar crest fibers, horizontal fibers, oblique fibers, apical fibers, and interradicular fibers. In the majority of these areas moderately thick collagenous bundles predominated; the interradicular group contained mainly thin collagen fibers. More reticular fibers were noted here than elsewhere in the ligament. In this group as in the preceding one, some collagen fibers were attached to alveolar bone, some were attached to cementum, and some were found to be in the intermediate area. The periodontal ligament was highly vascular with the majority of vessels extending into the ligament from the interdental and interradicular bone. The vessel walls were observed to contain argyrophilic reticular fibers.

A study of ground substance of the connective tissue of the periodontal ligament using AB-PAS stains revealed a predominance of violet staining fibers indicating the presence of acid mucopolysaccharides. The cell outlines were not prominent. The blood vessel walls showed an increase in acid mucopolysaccharides as indicated by the violet staining as compared with the 15 day specimens. The alveolar bone opposite the mesial root surfaces exhibited a wider band of blue staining recently apposed acid mucopolysaccharides than did other areas of alveolar bone. A thin blue line of newly apposed acid mucopolysaccharides was observed on all external cemental surfaces.

From 30 to 400 days, cementum was continuously added on all external root surfaces and was most marked at the apical region, where at 400 days each root exhibited a large bulbous mass of cellular cementum.

Collagen fiber bundles appeared to increase in thickness through 400 days; however, the interradicular collagen fibers were always less numerous and less dense than those in the rest of the ligament. The periodontal ligament exhibited a decreasing number of reticular fibers, and from 60 to 400 days these fibers were present almost exclusively in the walls of blood vessels; however, with silver stain a few delicate black

staining fibers could still be observed in the interradicular periodontal ligament at 400 days. Blood vessels were observed to enter the ligament from the interradicular and interdental bone in the 30, 60, 125 and 400 day specimens.

The majority of new bone formation, as evidenced by resting lines, was observed in the furcation area and alveolar crest. New bone formation was also observed opposite the mesial root surfaces and in the fundus. The majority of resorption of bone as evidenced by reversal lines and osteoclasts in Howships lacunae was observed in the alveolar bone opposite the distal surfaces of the roots. The above described bone changes could be observed through 400 days.

From 30 to 400 days the connective tissue ground substance contained both acid and neutral mucopolysaccharides as observed with AB-PAS stain. However, in all of these groups the violet staining acid mucopolysaccharides coating fibers seemed to predominate, and, furthermore, the intensity of the staining reaction appeared to increase with advancing age. The cementum in each specimen from 30 to 400 days exhibited acid mucopolysaccharides as seen by an outer thin blue staining surface layer. An outer blue staining band of acid mucopolysaccharides was also present on the surface of alveolar bone which was undergoing new bone formation. The

thickness of the blue line seemed to be proportional to the rate of new bone formation being thickest in the most active areas.

B. Autoradiographic Findings

1. Period of Root Formation

In the 10 and 15 day specimens the percentage of labeled connective tissue cells between the epithelial diaphragm and the fundus of the underlying bony crypt was relatively high compared with the percentage of labeled connective tissue cells in the periodontal ligament seen in the later sections.

In the 15 day specimens, as can be seen in Table I, the premitotic index was at its maximum value; 6.94% in the periodontal ligament on the mesial surface of the mesial root, 5.53% in the interradicular periodontal ligament between the roots, and 4.20% in the ligament on the distal surface of the distal root.

2. Period of Functional Occlusion

In the 30 day old animals there was a significant reduction in DNA synthesis in the connective tissue cells of the periodontal ligament as compared to the 15 day specimens.

The interradicular periodontal ligament between the roots exhibited a significantly higher frequency of labeling than did other areas of the ligament; 4.90% as compared to 2.63% on the mesial surface of the distal root.

In the 60 day old group, DNA synthesis in each of the previously specified areas of the periodontal ligament was significantly less than that observed in the 30 day old group. For example, the interradicular area showed an index of 2.55%. However, as observed in the 30 day old group, the interradicular periodontal ligament again exhibited a considerably higher frequency of labeled cells than did other areas of the ligament.

In the 125 day old group, only the interradicular area and the ligament around the distal root were studied, and in each of these areas, the percentage of labeled cells was less than that observed in corresponding areas of the ligament in the 60 day specimens.

As can be seen in Tables I and II the 400 day specimens continued to exhibit the presence of connective tissue cells capable of undergoing DNA synthesis. However, as compared with the 30 day specimens, there was a marked decrease in the number of labeled connective tissue cells in all areas of the ligament.

The periodontal ligament on the mesial surface of the mesial root (MMR) in the 400 day specimens exhibited a slightly higher percentage of labeled cells than did the corresponding area of the ligament in the 60 day specimens. The ligament on the mesial surface of the distal root (MDR) in the 400 day specimens also exhibited a slightly higher percentage of labeled connective tissue cells than did the corresponding area of the ligament in the 125 day specimens.

V. DISCUSSION

It is now well established that when cells double their DNA content prior to mitoses they take up various precursors; it is also well established that one of these precursors, namely thymidine, is incorporated exclusively into DNA. Therefore, if labeled thymidine were injected into animals, it would be incorporated into the DNA of those cells which were preparing to divide; these cells would then become labeled and would be easily located in autoradiographs.

In the present experiment, labeled connective tissue cells were observed in the periodontal ligament in each age group studied through 400 days, although there was a wide variation in the percentage of labeled cells in the different age groups, the fact remains that labeled connective tissue cells were present in the periodontal ligament of all animals in each age group. This suggests that connective tissue cells of the ligament are constantly being renewed by mitotic activity giving this tissue the capacity for growth and repair through at least 400 days of age.

These labeled connective tissue cells probably represent undifferentiated cells which are capable of differentiating into osteoblasts, cementoblasts and fibroblasts which are necessary in order to permit the continuous eruption of the rat molar described by Schour and Massler (1949) and the physiologic distal drift described by Sicher and Winmann (1944).

According to Schour and Massler (1949), prior to the occlusion of the molar teeth of the rat, the rate of eruption is very rapid, but as soon as antagonism is established the rate is markedly retarded. This difference in eruption rates seems to correlate well with the findings on a cellular level in this experiment. As can be seen in Table I, it was in the developing periodontium of the 10 and 15 day old specimens that the premitotic indices reached their maximum values. This high frequency of labeling was observed in that portion of the dental follicle located between the epithelial diaphragm and the underlying bony crypt in the 10 and 15 day old specimens. It is assumed that this proliferation center provides cells which differentiate into the cementoblasts, fibroblasts and osteoblasts of the periodontium in the rapidly erupting rat molar. Hoffman and Gillette (1962) described a similar growth center in the developing roots of hamster molars. In addition to this apical growth center, a high frequency of labeling was also observed in the developing

vertical and interradicular periodontal ligament in the 15 day old specimens.

At 30 days of age the rat molar is in functional occlusion and the rate of eruption is, therefore, biomechanically reduced. It would seem reasonable to assume that once the teeth are in functional occlusion the need for dividing and differentiating cells would be reduced. This assumption seems to be borne out by the results of this experiment. As can be seen in Table I there is a sharp reduction in the number of labeled connective tissue cells in the periodontal ligament of the 30 day old group as compared with the younger age groups.

After the first molar reached functional occlusion, the interradicular periodontal ligament always exhibited a higher percentage of labeled cells than did other areas of the ligament. This finding correlates well with the observation that the majority of new alveolar bone was deposited in the furcation in all age groups studied. These findings suggest that the rat molar teeth move at a greater rate of speed in an occlusal direction than in a distal direction through at least 400 days.

It can be seen in Table I that there is some individual variation in the percentage of labeled cells in the vertical

sections of the periodontal ligament in each age group studied. This is to be expected for tooth movement is intermittent and nonuniform; hence, at any given moment areas of alveolar bone resorption alternate with areas of reparative apposition. Thus, variations in the number of new osteoblasts, fibroblasts and cementoblasts both within different areas of the ligament of the same individual as well as variations in corresponding areas in different individuals of the same age group would be the rule rather than the exception.

Although the total percentage of labeled cells in the periodontal ligament as a whole decreased progressively with aging, specific areas of the ligament did not exhibit a progressively decreasing premitotic index. For example, the periodontal ligament on the mesial surface of the mesial root (MMR) in the 60 day old specimens exhibited an index of 0.38%, whereas, the corresponding area in the 400 day old specimens exhibited an index of 0.54%. Since the movement of teeth is intermittent and nonuniform, and since these movements continue throughout the lifespan of the animal, it seems reasonable that the higher premitotic index in the 400 day specimens represents an area of active reparative apposition, whereas, the lower index in the 60 day specimens represents more of a steady state maintenance rather than an area of active apposition.

It can be seen in Table II that as the animals in this experiment increased in age from 30 days to 400 days there was a sharp reduction in the percentage of labeled connective tissue cells. It would thus appear that one of the manifestations of aging, at least in the periodontal ligament of the rat molar, is a decrease in the total number of connective tissue cells undergoing DNA synthesis, and, hence, mitoses. As a consequence, one might expect that the cells of the periodontal ligament would be able to repair the wear and tear of daily function less rapidly with advancing age.

The connective tissue fibers of that portion of the dental follicle located between the epithelial diaphragm and the underlying bony crypt of the 10 and 15 day specimens consisted predominantly of fine irregular coursing reticular fibers. The vertical portion of the developing periodontal ligament of the 15 day specimens exhibited reticular and collagen fibers; however, the collagen fibers greatly outnumbered the reticular fibers. In no instance could individual fibers be traced through the entire width of the ligament. Some of the collagenous fibers were attached to cementum; some were attached to bone; and, a third group was found to be intermediate, appearing to connect the other groups in the central portion of the ligament. On the basis of this finding, this author tends to agree with Sicher (1954) on the

existence of an intermediate plexus.

In all areas of the periodontal ligament studied there appeared to be a decrease in the number of reticular fibers, and an increase in the number and thickness of the collagenous fiber bundles with increasing age. These findings are in agreement with the changes observed in other connective tissues during the aging process. Gross (1950) discussed the predominance of argyrophilic reticular fibers in the skin of the newborn rat which changed gradually with aging into collagenous fibers. Barnfield (1955) found that collagen fibrils in abdominal skin samples increased in width with advancing age. Asboe-Hansen (1963) concluded that the fibrillar density is increased as connective tissue becomes older.

It is interesting to note that the interradicular periodontal ligament always exhibited a greater proportion of reticular to collagen fibers, and that the collagen fiber bundles in this location were always less dense than those seen in other areas of the ligament.

In all age groups studied, the periodontal ligament appeared to be well vascularized, and contrary to the findings of Bernick (1960), blood vessels were observed to enter the ligament from interdental and interradicular bone in rats 400 days of age. One factor in this difference might be the

difference in the strain of rats used. The importance of considering the possible difference in various strains of the same species was pointed out by Belting, Schour and Weinmann (1953).

Apposition and resorption of alveolar bone were evident throughout the duration of this experiment. The majority of new bone formation appeared to be in the furcation area and at the alveolar crest. Smaller amounts of new bone apposition were also observed opposite the mesial root surfaces and in the fundus. The majority of bone resprption appeared to occur opposite the distal root surfaces. The apposition and resorption of bone did not appear to be continuous but were rather intermittent processes. Cellular cementum was also apposed on the root surface through 400 days. These findings are in agreement with those of Sicher and Weinmann (1954) who described a physiologic distal drift of rat molar teeth, and with the findings of Hoffman and Schour (1940) who concluded that the apposition of alveolar bone of the crests and fundi and of cellular cementum is a continuous process throughout the life of the rat.

In the ground substance of the periodontal ligament studied with alcian blue and periodic acid-Schiff stains the presence of both acid and neutral heteropolysaccharides was

observed through 400 days. Since these tissues were fixed in formalin it is quite probable that the water soluble mucopolysaccharides were lost during the period of fixation. However, the water insoluble, presumably highly aggregated, mucopolysaccharides which remained in the tissues tended to exhibit a predominance of violet staining acid mucopolysaccharides in all animals 30 days of age and older. Furthermore, the intensity of the violet stain appeared to increase progressively with age. It thus appears that as the rat gets older there is a progressive increase in the amount of water insoluble presumably highly aggregated acid mucopolysaccharide in the ground substance of the periodontal ligament. This finding correlates well with the suggestion of Gersh and Catchpole (1960) that with increasing age there is a relative increase in the denser (colloid-rich) phase of the ground substance. In this respect then, the ground substance of the periodontal ligament appears to react to the aging process in a manner analogous to that of the connective tissue ground substance in general.

Since one important function of acid mucopolysaccharide is to act as a cementing substance holding collagen fibers together, one would assume that an increase in collagen fibers would be accompanied by a corresponding increase in acid mucopolysaccharides, as was observed in this experiment.

One might postulate that although the fiber to gel ratio increases with aging the ratio of collagen fibers to their acid mucopolysaccharide cementing substance remains constant.

With the combined alcian blue periodic acid-Schiff stains, a blue staining band was observed on all cemental surfaces, and on many alveolar bone surfaces. The presence of this band was observed most consistently on all cemental surfaces, and on the surfaces of alveolar bone in the furcation area, at the alveolar crests, and opposite the mesial root surfaces. Furthermore, these same surfaces tended to exhibit a somewhat wider blue staining band in the younger age groups. It is suggested that this staining reaction represents the presence of water insoluble presumably highly aggregated acid mucopolysaccharide in the ground substance of newly deposited cementogenic and osteogenic connective tissue.

Since the color and intensity of the blue staining band tended to remain constant in all areas with aging, and since the width of the band tended to decrease with aging, it appears that the quantity and not the quality of this particular fraction of the ground substance of cementogenic and osteogenic connective tissue is affected by the process of aging.

VI. SUMMARY AND CONCLUSIONS

Fifteen rats aged 10 to 400 days were injected intraperitoneally with triated thymidine and sacrificed one to two hours after injection. Mesiodistal sections were made through the molar region of the right maxilla 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 prepared on the hematoxylin-eosin-stained slides. The percentage of labeled connective tissue cells in the periodontal ligament was determined.

On the basis of the general histologic and radioautographic studies, the following conclusions were made:

1. The connective tissue of the periodontal ligament has the capacity for growth and repair through at least 400 days.

2. That portion of the dental follicle located between the epithelial diaphragm and the underlying bony crypt acts as a growth center for the periodontal ligament, cementum and alveolar bone in the erupting first molar.

3. In the periodontal ligament the aging process in some as yet unexplained way results in an overall steadily decreasing number of DNA synthesizing connective tissue cells.

4. An intermediate plexus is present in the periodontal ligament of the rat molar.

5. There is a decrease in the number of reticular fibers and an increase in the number and thickness of collagenous fiber bundles in the connective tissue of the periodontal ligament with aging.

6. There is a progressive increase in the amount of water insoluble acid mucopolysaccharide coating the collagenous fibers of the periodontal ligament with aging.

7. The periodontal ligament is well vascularized through 400 days, the primary source of the blood supply being from branches of the interdental and interradicular arteries.

8. Bone and cementum apposition occurs through 400 days and is most active in the younger age groups.

9. The rat molar continues to erupt as well as to drift in a distal direction through 400 days.

10. The osteogenic and cementogenic connective tissue contain water insoluble acid mucopolysaccharides which decrease quantitatively but not qualitatively with aging.

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VIII. APPENDIX

% Labeled Connective Tissue Cells

Age Group and Number of Animals Per Group

	MAF	HF	DAF	MMR	DMR	I	MDR	DDR
10 Day								
#1	4.80	2.94	1.90					
#2	6.80	6.63	4.50					
#3	4.93	4.40	3.76					
Mean	5.53	4.66	3.06					
15 Day								
#1	5.26	4.25	3.94	6.84		6.10		4.44
#2	5.41	4.78	4.13	7.80		5.90		5.00
#3	5.11	4.13	3.77	5.44		4.55		3.12
Mean	5.26	4.36	3.95	6.94		5.53		4.20
30 Day								
#1				1.14	1.40	4.87	2.14	1.72
#2				1.02	1.44	4.68	1.96	1.63
#3				1.55	1.93	5.20	3.73	0.76
Mean				1.24	1.59	4.90	2.63	1.37
60 Day								
#1				0.54	0.62	2.85	0.55	0.64
#2				0.21	0.60	2.20	0.60	0.98
Mean				0.38	0.61	2.58	0.58	0.81
125 Day								
#1						1.04	0.01	0.60
#2						2.50	0.36	0.70
Mean						1.77	0.18	0.65
400 Day								
#1				0.76	0.13	0.39	0.51	0.15
#2				0.32	0.25	0.58	0.07	0.06
Mean				0.54	0.19	0.48	0.29	0.10

TABLE I

Mean Percentage of Labeled

Connective Tissue Cells

Age Group	MMR	DMR	I	MDR	DDR
30 Day	1.24	1.59	4.90	2.63	1.37
400 Day	0.54	0.19	0.48	0.29	0.10

TABLE II

FIGURE 1. Labeled connective tissue cells in the vertical portion of the developing periodontal ligament at 15 days of age. (H & E stain x 400)

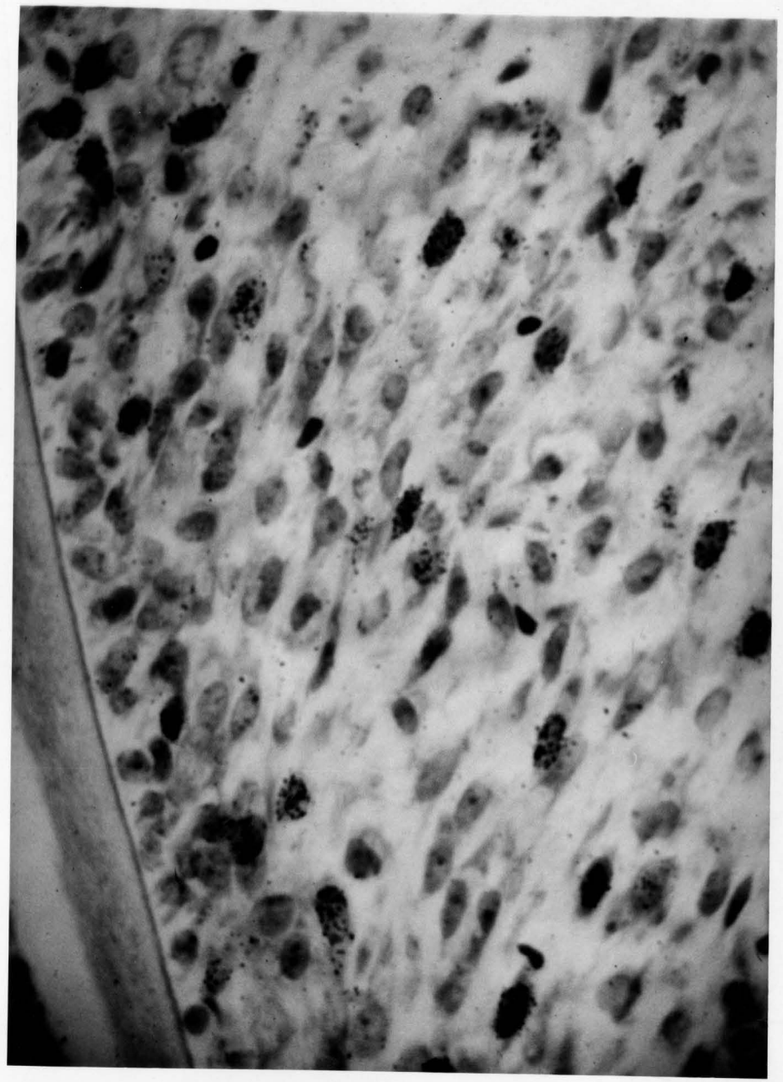

FIGURE 1

FIGURE 2. Labeled connective tissue cells in the interradicular periodontal ligament at 400 days of age. (H & E x 400)

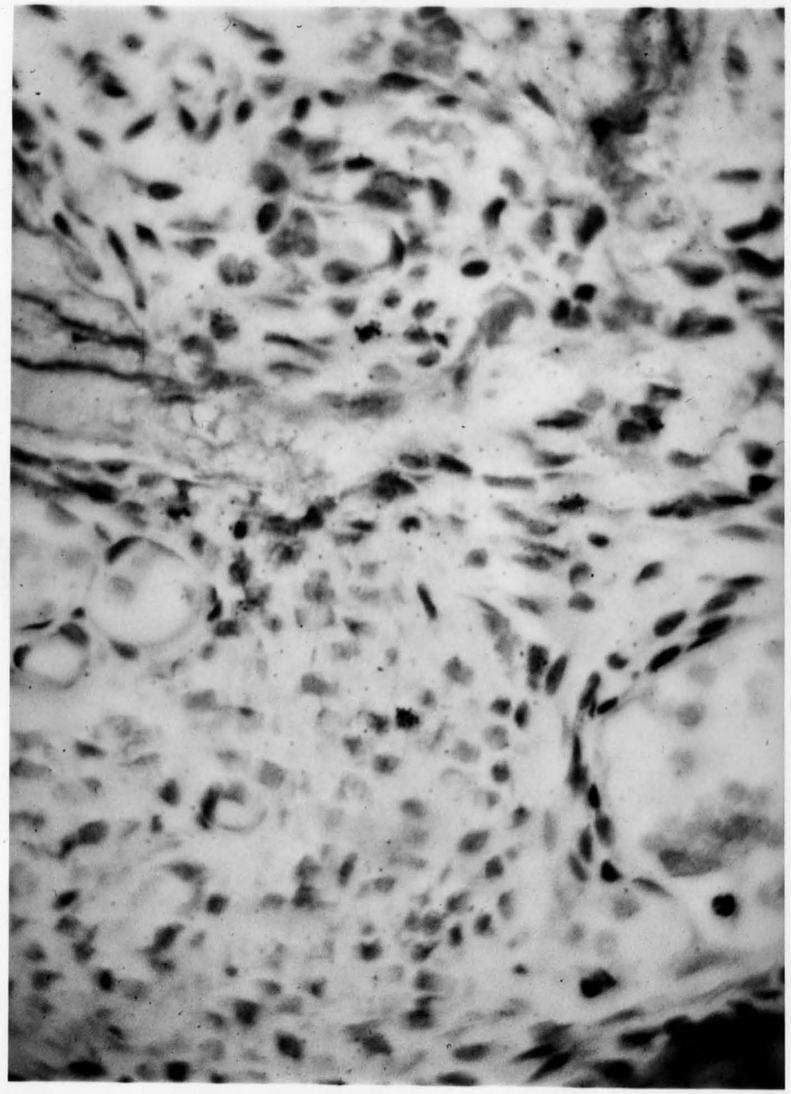

FIGURE 2

FIGURE 3. Fiber bundles of the periodontal ligament at 30 days of age. (Heidenhain's Azan Stain x 125)

FIGURE 3

FIGURE 4. Periodontal ligament at 400 days of age exhibiting increased density of fiber bundles. (Heidenhain's Azan Stain x 125)

FIGURE 4

FIGURE 5. Interradicular alveolar bone at 400 days of age exhibiting apposition lines. (H & E Stain x 125)

FIGURE 5

APPROVAL SHEET

The thesis submitted by Jerald L. Jensen has been read and approved by three members of the faculty of the Graduate School.

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.

DATE: May 16 1966

Patrick D. Toto, D. D. S., M. S.

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Signature of Advisor