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# Utilization of collagenase as an aid in visualizing the innervation of the periodontal tissues

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# BOSTON UNIVERSITY SCHOOL OF GRADUATE DENTISTRY

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# THESIS

UTILIZATION OF COLLAGENASE AS AN AID IN VISUALIZING THE INNERVATION OF THE PERIODONTAL TISSUES

by

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School of Dentistry

submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry (Periodontology)

54,502

#### **Boston University**

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# ABSTRACT

Mandibular incisors and contiguous structures of Sprague-Dawley rats were fixed in Carnoy's solution, decalcified in 5% nitric acid, dehydrated, embedded in paraffin and serially sectioned at 10-15u. Representative slides of the periodontium were chosen and stained with Goldman-Bloom Trichrome, or a modified Gordon-Sweet Silver stain, both with and without prior collagenase digestion. The enzyme solution contained 5mg collagenase (125-200 units/mg) per lcc buffer solution and was incubated with the slides at 45°C for 33 minutes. Both groups of slides were then colloidinized and stained. In undigested sections nerve fibers and endings were masked by collagen and their relationships could not be discerned. In enzymatically treated sections terminal nerve fibers were seen to course with capillaries creating an arcuating rete in the subepithelial zone of the gingiva. Nerves were not seen to penetrate the epithelium. At the alveolar crest arborizing terminal coils appeared to encircle the area occupied by the Transseptal or Group C fibers and seemed to terminate as free nerve endings. On the alveolar side of the periodontal ligament the nerve fibers appeared to encircle Sharpey's fibers while on the cemental side individual nerve fibers paralleled the bundles and ended as knob-like structures or as free nerve endings.

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# INTRODUCTION

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It has been observed that with routine histologic staining methods the dense collagenation of the periodontal apparatus hinders visualization of existing neural elements. As a means of overcoming this problem, Bernick (1-4) has employed several nonspecific proteases such as pepsin and trypsin to remove most collagen and reticulin from his sections in order to delineate innervation. He has found that these proteases suppressed the staining qualities of the two scleroproteins so that stained nerve fibers were seen against a poorly stained background.

It is the purpose of this paper: to describe a method of specific tissue digestion in decalcified rat mandible utilizing a collagenase; to demonstrate the neural elements of the periodontium of the continuously erupting rat incisor; and to illustrate the extent of Sharpey's Fibers insertion into the alveolar plate of bone.

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# REVIEW OF THE LITERATURE

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# GINGIVAL INNERVATION

The innervation of the gingiva is derived from fibers of the labial or lingual branches of the Second and Third Divisions of the Trigeminal nerve and to a much lesser extent, from anastomosing fibers from the periodontal ligament (2,5-9). The major nerves, coursing with the blood vessels in the supraperiosteal area of the alveolar plate, form a rete which has been termed the "deep plexus" (10). As these fibers pass through the connective tissue of the gingiva, branches are given off to terminate as a "superficial plexus" (10) in the lamina propria of the dermal papillae (2,11) and on occasion continue into the epithelium as fine ultraterminal fibers (4-6,8,12-14). The interproximal gingiva is innervated by coronal extensions of the nerve plexii of the periodontal ligament (4) as well as from supracrestal ramifications of the interdental nerves terminating in the transseptal fiber systems of adjacent teeth (4,16). Bernick (2,4,8) has reported that he did not observe specialized endings such as Krause's or Meissner's corpuscles in his 1% pepsin preparations of the gingiva. Simpson (15) has also noted this negative observation. Other writers (5,11,16-20), however, have reported observing these corpuscles and other complex end organs in the gingival tissues. Such absence or sparcity of organized and specialized nerve endings is not unusual as it has also been observed in areas of skin and mucous membrane which exhibit varying degrees of sensibility

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# INNERVATION OF THE PERIODONTAL LIGAMENT

The function of the periodontal ligament nerves is to transmit impulses of resultant forces of occlusion and mastication (touch, pressure and pain) to higher neurologic centers where appropriate responses can be transmitted to the effector muscle groupings to elicit protective reactions. The prime sources of this afferent innervation are: periodontal branches of the Dental Nerve after it perforates the alveolar plate before entering the tooth (4,6,15,16,19,21-23) and from the Intra-alveolar Nerve as it courses crestally and its lateral branches perforate the cribriform plate. In the alveolar phase of the periodontal ligament the two groups anastomose and send branches both apically and occlusally to form a complex rete parallel to the long axis of the tooth. From this network branches are given off to terminate in the connective tissue. Rapp and co-workers (23) have felt that the larger neural branches run in association with the blood vessels while the smaller nerve fibers may not. The intraseptal nerves provide the major portion of the ligament innervation and as a consequence, surgery at the apical area of the tooth or inflammatory destruction of the tissues of this region does not greatly compromise the nerve supply of the rest of the periodontal ligament. The nerves may pass close to the cementum and alveolar bone and their peripheral endings may form fine arborizations. Sicher (19) has described three types of observed endings: knob-like

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structures; rings or loops around principal fiber bundles; and free nerve endings. Simpson (15) has commented that definite end organs are seldom seen in the periodontal ligament and that fine endings are the predominating terminal feature. Studies by Bernick (4) in 1959 also have reported the finding that Meissner's, Ruffini's or Pacini corpuscles are not observed. In the apical third of the periodontium of a human tooth myelinated nerves have been seen to lose their sheath and terminate as spindle-like structures (4,16). Brashear (24) has felt that as the coarse nerve fibers travel occlusally in the alveolar phase, sensations are mediated by the varying caliber of myelinated and nonmyelinated fibers present: pain conducted by small diameter nerves of both types; temperature by intermediate medullated fibers; and touch by the large myelinated nerves. Nerves may form rings or loops around principal fiber bundles or may terminate between them so as to function in proprioception and localization (5,25). In the cemental phase, Rapp, Avery, and Rector (23) have observed that most neurofibrils approach the cementum to form loops and then turn back towards the mid-portion of the ligament. van der Sprenkle (25) has described a rete of nonmedullated fibers coursing radially from the central area of the periodontal ligament to penetrate the cementum.

The coronal termination of the nerve fibers of the periodontal ligament has been reported in the circular fiber group

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of the gingiva where they anastomose with nerve fibers of adjacent teeth to contribute to the total innervation of the area (16).

# NEURAL ELEMENTS OF THE PERIODONTIUM

Nerves of the periodontium, after branching from the main neural trunks, course with the blood vessels and may ramify as simple or organized endings in connective tissue. In the epithelium of moist mucosa, fine ultraterminal filaments have been observed by Gairns (5) with the electron microscope.

The tendency for varicosities along the length of nerves has been clearly demonstrated with Schabadasch's technique for vital neural staining with methylene blue (13,26-28). In this method tissue is exposed to 0.01% buffered methylene blue dye for 20 minutes, during which time there is selective neural absorption of the material. If however the tissues are stained for a longer period, they undergo morbid changes and indiscriminate staining of all structures occurs. O'Leary and co-workers (28) have employed a 6% solution and increased oxygen tension in supravital brain preparations to maintain coloration of the methylene blue until a mordant solution could be used to render the dye insoluble. In general, concentrations below 1% have been used postvitally, while higher concentrations and smaller volumes have been used for subcutaneous, intraperitoneal, intravenous or intra-arterial injection.

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In studies by Dixon (13) in 1963 and by Sakada and Maeda (29) in 1967 the innervation to the oral mucous membranes and periosteum have been demonstrated in thick sections. The presence of a fine subepithelial nerve plexus and a coarser deep dermal plexus in the dorsum of the tongue, hard palate, buccal mucosa and the floor of the mouth of various animals have been described. It has also been noticed by Dixon (13,30), Tolman (20), and Winkleman (31) that the tissues in the anterior portion of the mouth were more highly innervated than those in the posterior. The deeper plexus gave rise to branches which ramified as fine free or organized terminal endings in the dermal papilla (12). Often a single area has been observed to have multiple innervation through fibers originating from several points (13). This arrangement which allows for accurate tactile localization (33) is similar to that seen in the skin and has been described by Kuntz (32) in 1938. Nerve fibers have been found to course with the arterioles, venules and many of the capillaries. The subepithelial plexii discerned are primarily terminal ramifications of afferent fibers from the deep plexus. Where dermal papillae are absent or poorly developed, intraepidermal nerve filaments may been seen which originate from large diameter myelinated fibers of the superficial plexus (5,12,13,25).

Another method of demonstration of nervous elements has been localization of cholinesterase activity (34-39). This

In shuites by Dixon (13) in 1969 and by Saturda mi -Meads (29) In 1967 the impervation to the oral whole new branes and periosteum have been demonstrated in thisk sections. The presence of a find an entitlelial parve virtue and a convert deep deried plants in the dorein of the balance, hard palate, buotel mucosa and the flam of the south of weed only und distributed descriped. It has also been noticed by Dixon (13, 30), "plann (20), and Minkleman (31) that the tlanues in the shterior cofficer of the contract ware adre highly innervated than these in the porterior. The deater plants gave has be branches which raiding as fine (St) all organized end at manthes leaterst bestmant or earth often a single area has been observed to have autoinite innervetion through fibers originating from ceveral roints (13). This arrangement which allows for somethe testils Logalization (33) is similar to that aren in the ship and inter been described by sumtry (32) in 1936. Serve finers have been found to course while the enteriols, vanides and sany of the copiliaries. The suborithalial playit distruct in and contents thereine in another to strong the set all there in a the deep plexus. Meare dermal papillie are abaent or peorly deteloped, intraceptderail narva filements may bush is an which orderingte from Large diageter avelignered forte of the Augers fictal plenes (5,12,13,25).

Another zetroi of demonstration of nervous elements has been localization of anoliaesterese activity (34-39). This

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enzyme in the body functions to inactivate acetylcholine and thus terminates conduction of neural impulses along neurofibrils and at motor end plates. A "specific" esterase is primarily found in neural tissue and a "nonspecific" type is present in blood serum. By pretreating tissue sections with di-isopropylfluorophosphate before incubation with a reactive substrate it has been found that the nonspecific cholinesterase was inactivated and sites of the specific enzyme were clearly demarkated (34-36); and thus the nerve paths could be followed in tissues. The sites of enzyme activity were visible as dark brown deposits of copper sulfide (37).

To differentiate sensory from autonomic innervation, sensitization of adrenergic fibers is possible with subsequent visualization by fluorescent microscopy. Anneroth (40) and Pohto (41) have demonstrated the adrenergic innervation in freeze-dried human pulpal tissue. Laties and co-workers (42), using buffered paraformaldehyde, have fixed cryostat sectioned cardiac tissue and have found that this technique produced results as acceptable as the freeze-dried method. ARGYROPHILIC STRUCTURES

Silver impregnation has been a technique that is widely used is neurohistology (43-46). Various silver and connective tissue stains have also been employed to demonstrate nerves in the periodontium as well as collagen fiber groupings. The black coloration of argyrophilic structures is produced by

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Silver lugremation has been a teamingle their is widely need in neurohistology (+)-+6). Verious cilves and conductive these stains have also here relayed to demonstrate betwee in the periodonitum as well as collared the demonstrate betwee in black coloration of argumentic structures is melaced by ...

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the precipitation of silver salts. Collagen, reticulin, and nerves are argyrophilic with collagen appearing golden-brown and reticulin and neural elements, black (47). The similarity in staining quality complicates the identification and characterization of these three structures in the periodontium. A morphologic comparison reveals: that collagen and reticulin are more tortuous in their courses than nerves; they do not exhibit the varicosities observed on neural elements (48); and they do not form specialized structures on their terminus. Reticulin, however, forms a finely branched rete throughout the gingiva which is very evident in the subepithelial zones and may be mistaken for nerve fibers. To further confuse matters, wavy unbranched, argyrophilic collagen fibers may also be present as a result of a healing wound (49).

# FIBER APPARATUS

The normal periodontal unit is replete with an extensive collagen fiber system which functions: to support the gingival tissue, closely approximating it to the tooth and in doing so minimizing the stresses induced in mastication (50); to act as a mechanical block to the apical migration of the epithelial attachment (51); and by their arrangement act as a barrier to inflammation.

The function of the fibers of the attachment apparatus is to furnish support, assisting the dynamic hydraulic mechanism (12,52,53) to dissipate occlusal loading pressures, and to anchor the teeth to the alveolar bundle bone.

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These collagenous fibers in their insertion in bone and cementum are known as Sharpey's Fibers. In the periodontal ligament they do not span the entire width (51,53-56) as was once believed (57), but many fibers seem to be employed in the fabrication of apparently singular traversing bundles. Fibers have also been seen to decussate from bundle to bundle forming an involved plexus between them (54). These fibers are inelastic and quite wavy and it has been suggested that the straightening of these undulations is part of the mechanism of force dissipation (54,57). In the mid-portion of the ligament an area of intertwining of alveolar and cemental phase fibers (the intermediate plexus) has been described by Goldman (51), Stallard (56), and Sicher (55). It has been suggested that this arrangement of fibers allows for a more rapid turnover and orientation of alveolar phase fibers in response to physiologic demands, as well as to dissipate acute forces to the teeth (26).

The proximal ends of Sharpey's Fibers originate in the periodontal ligament and terminate either in the cementum of the tooth or bundle bone of the cribriform plate of the alveolus. Bone is laid down in a lammelar fashion without regard to the orientation of these fibers. While Sharpey's Fibers can be demonstrated in association with the interstitial lammellae of bone, they are not seen in haversian lamellae. This, Provenza (54) has felt, confirms their periosteal origin.

These colleranous fibers in their insention in bone and cementum are mount as Sharpey's Fibers. In the particion tal. ligeners they do not cran the active width (51, 33-56) and once believed (47), but mont fibers still to be employed in the fabrication of apparently sincular traversios in molice. Miners have also been as decourtants from house is reading forming an involved places between them (54). Three fibers And becomments many it is the way of the bas beau summersed shat bhe stratghtentag of these monthations is part of the mechanicad of forde. dissignation (54, 52). In the wid-portion of the lighted an area of intertwinise of slyeolar and cemental phase fleers [the intermediate plarma) has been described by Goldman (21), Stallard (26), and Micher (55), It has been succested that this errangerent of filese allows for a more rapid turnover and erlentetion of alveolar phase fibers in responde to physiclotic demands, at well do to diasignts acuts foress to the testh (25). The proximal ands of Sharpey's Fibers originate in the periodental lisement and tempinete either in the community the tooth or bundle bone of the oribritors miste of the alveolus. Sone is laid doon in a lavaalar fachton without repart to the orientation of these fibers. Bille Sharesets Ethers can be demonstrated in sesociation with the interviet-"thal Iscondleo of bone, shey are not seen in neversion landlise. This, Evoyanza (54) had reit, confrint their port-. Winters Landso

Bundles of collagen enter the cementum and bone at or nearly right angles and disaggregate as splayed out fibrils which are oriented in, and obscured by the bony matrix (54,58,59). Plano-parallel sections at the periodontal surface of these two tissues reveal that most fibers have diameters of less than 10u but some are seen to exceed 20u. Inoue and Akiyoshi (60) have correlated this fiber diameter variance and quantity per unit area to the functional demands that are placed on the periodontium.

In both these ossified structures Sharpey's Fibers demonstrate an uncalcified core 0.2-0.5u in diameter, abruptly becoming calcified at the periphery. Selvig (61) has noted that fibers were occasionally crystallized in their entirety. The crystallites of hydroxyapatite are arranged so that the c-axis is parallel to the c-axis of the collagen fibrils (62,63) but randomly distributed around them (16). This arrangement of crystals to the collagen fibrils of Sharpey's Fibers is similar to that found between the inorganic salts and matrix of bone.

The various periodontal ligament fiber groups transmit vertical and angular forces to the cribriform plate of alveolar bone where the internal bony trabeculae of the jaws form trajectories to direct this energy away from the masticatory system (51,54,64,65).



Bundles of collegen enter the demonton and bone at or rearly right contents and disacersects as shared out (ibrild which are priorid in, and obseured by the boxy matrix (Sh.58.59), Plano-perallel sections at the periodottal are face of these two tissees reveal that set fiber share disactors of less than its but sens are note to enced 200. Facus and Ativochi (60) have correlated this fiber disactor that are placed on the periodottics.

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# MATERIALS AND METHODS

Thirty Sprague-Dawley rats of either sex, weighing 250-400mg were sacrificed by excessive inhalation of anhydrous ethyl ether in a closed container. Bucco-lingual cuts were then made through the mandible distal to the continuously erupting incisors followed by sharp dissection at the base of the labial and lingual vestibules severing all muscle and mucosal attachments to the jaw. This allowed for in toto removal of the tissue under investigation. Fixation was accomplished in Carnoy's solution for 18 to 24 hours, after which the specimens were washed in water for one hour and decalcified in 5% nitric acid for 3 to 4 days. The tissues were then washed and dehydrated in ascending alcohols, embedded in paraffin, serially sectioned labio-lingually parallel to the saggital plane on an International Minot Rotary Microtome at 10-15u, affixed to slides using egg albumin (J. T. Baker Chemical Co., N. J.) and allowed to air dry. Representative sections demonstrating various areas of the tooth and periodontium were selected, deparaffinized in xylene, hydrated in descending alcohols to be stained with a modified Gordon-Sweet Silver stain or Goldman-Bloom Trichrome stain for connective tissue, both with and without prior collagenase digestion. The collagenase solution was prepared according to Mandl, MacLennan and Howes (66,67) utilizing lcc of phosphate buffer solution (M/15 Na2HPO4 and M/15 KH2PO4 in an 0.9% NaCl solution (see Appendix  $\underline{B7}$ ) adjusted to a pH of 7.4

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which contained 5mg of the enzyme (125-200 units/mg, Nutritional Biochemical Corp., Cleveland, Ohio). In an attempt to minimize differences in units per milligram between enzyme lots, the digestion solution was prepared in 200cc aliquots. 5cc portions were then placed in plastic vials and frozen until needed. Collagenase solution compounded in this manner retained its potency for six months.

Each tissue section was incubated with 0.2cc of the above mixture (68) at 45°C for 33 minutes, over water in a sealed container to maintain hydration. They were then washed in distilled water, dehydrated in ascending alcohols, rinsed in anhydrous ethyl ether and absolute alcohol (1:1), colloidinized in 1% Parloidion (Mallinckrodt Chemical Works, N. Y.) in equal parts of anhydrous ethyl ether and absolute alcohol for one minute, air dried and placed in 70% alcohol. Those sections stained with Gordon-Sweet's method were carried through silver staining, and the steps involving toning with yellow gold chloride and removal of Parloidion were omitted. All slides were then rinsed in xylene and mounted in Permount. Sections used as controls were not subjected to collagenase digestion but were incubated with distilled water along with the experimental group and stained with the enzyme-treated tissues.

Color transparencies of representative areas at 100x, 430x and 1000x (oil immersion) magnification were made utilizing a Honeywell Spotmatic 35mm single lens reflex

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camera body adapted to a Leitz Ortholux microscope with achromatic objectives. Illumination was color corrected with an 82C Wratten filter to 3200 K. The Ektachrome X Type B film employed was processed by Kodak Processing Laboratory (1712 South Prairie Avenue; Chicago, Illinois). Using a 4" x 5" camera housing adapted to the Leitz Ortholux microscope 4" x 5" transparencies with Ektachrome Type B film were obtained. These were processed into lantern slides by Linhoff Color Processors (Minneapolis, Minnesota). Color prints  $(3\frac{1}{2}$ " x  $4\frac{1}{4}$ ") were then made from both the 35mm and 4" x 5" transparencies on Kodak Type C paper.

Control alidas (these not treated with collingeners) and stated with Conton-start filter State or Collingen down Fichness), demonstrated cornal histologic erstem, here, optibaling various components of fiber excernits, and macular dimensis were realily identificable, although colleher detail was lanking (Mar. - 1, 2, 14, 21, 22). No. 10. here retioning eveness (Mar. 12, 14, 21, 22). As a consections of allows scaling, college conserved solder to dimensions without reterms and a sector of the sector dimension end with theorem state, have (Mar. 22). The advancement of the apithelics, reterms and the sectors allowed and silver. In the area and interaction of elan wollder to dimension of the apithelics, reterms and the sectors of the base dominant allows and the file, 2, The reterminant base dominant fibers are abserved (Mar. 2). The reterminant base ormera body wherea to a leate bytholyg disrocope with extrements of jestives. Illusination was color corrorted with an 200 createst filter to 3200 K. The Estacheme X Type is film septored was processed by Rodessing laboratory (1712 South Preivie Avenue Chicaso, 1111aois). Iting a by g or ensert homeing Adapted to the Leibe Cribolay along the star processed to the Leibe Cribolay along the star processed into Lange Cribo calides by Liveor the Processed into Lange slides by Liveor Ford Ford Ford Star (Strange) a color primes (300 x 44°) were then ands from both the list in and by y transporter on Kodek Type C paper.

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#### RESULTS

# GENERAL OBSERVATIONS

In the development of this technique, several critical observations were made. The residual collagen matrix of both the decalcified tooth and bone appeared to be missing upon microscopic examination or was observed floating as fragmented sections when all slides were removed from the incubating chamber. Tissue sections subjected to digestion for longer than 33 minutes at temperatures higher than  $45^{\circ}$ C excessively lost component connective tissue structures resulting in so drastic a disturbance of the anatomical relationships that these slides were not of histologic value. <u>CONTROL SECTIONS</u>

Control slides (those not treated with collagenase, but stained with Gordon-Sweet Silver Stain or Goldman-Bloom Trichrome), demonstrated normal histologic anatomy. Tooth, epithelium, various components of fiber apparatus, and vascular channels were readily identifiable, although cellular detail was lacking (Fig. 1-3,12,14,21,22,27,30-33). The neural elements, however, were masked by the dense collagen and reticulin systems (Fig. 12,14,21,22). As a consequence of silver staining, collagen appeared golden to black-brown, and with trichrome stain, blue (Fig. 22). The subepithelial reticulin rete was quite pronounced in sections stained with silver. In the area immediately subjacent to the basal cells of the epithelium, a dense network of black staining fine fibers was observed (Fig. 2). The rete of reticulin was

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In the development of this termique, ceremal critical observations were made. The realdeal collegen matrix of both the decaled field tooth and here ensained to be sizaing upon microscopic excitatation of was observed filesting as fragmentid rections when all allow were removed from the incubating charder. Sizawa sections subjected to dignation for longer than 31 almotes at tampenetures higher than 50° for longer than 31 almotes at tampenetures higher than 50° resulting in as static comments to the material relationships that there alloes were not of historical contract sections of the material

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most evident in those sites where digestion was incomplete, and this fiber group was viewed as a discrete accumulation of black punctate bodies (Fig. 9).

In the labial marginal gingiva it was observed that the larger vascular channels were found in the midportion of the lamina propria -- between the sulcus (or its ameloblastic apical extension) and the labial epithelium. From this central area vessels branched laterally to their terminal circulatory beds. The gingival fiber system of this area appeared to be oriented parallel to the odontogenic and sulcular epithelium while in the facial phase it presented an arborizing configuration (Fig. 6). At what appeared to be the base of the sulcus (the enamel being dissolved), a deeply penetrating dentate epithelial downgrowth was observed in all sections (Fig. 6). This erratic formation became an orderly rete peg arrangement of the ameloblastic layer farther apically. A hexagonal ordering of these cells was apparent when viewing them at right angles to their secretary surface.

The labial plate of bone was very thin and demonstrated perforating vascular channels from the periodontal ligament anastomosing with those of the labial periosteum (Fig. 27-29).

Major blood vessels were also observed in the mid-portion of the lingual gingiva. In the periosteal area vascular channels of finer caliber were noted (Fig. 2).

Linear argyrophilic structures appeared to originate in the mid-portion of the periodontal ligament. This may be the intermediate plexus area or an area of fiber reorientation.



most evident in those sites where direction was incomplete, and while fiber erous was viewed as a discrete modifi did bra of black pimetable bodies (Fir. 9). In the laboral marginal sincity is was observed that the larger varoular observed ever forma in the reducertion of the lating propris -- between the sulcus (or i'ts and d'lantin apical extendion) and the latial and the indicative fairs control great villageted bestand alegues to train terminal. - Ins pro of mercet to be and of le liented be weekto ad of betweetoe on betweende at eagle Delaar ond at slide suifeddere teine arbort sine coolt meation (Fig. 6). At what appeared to be the bane of the relevant (the enged) being dissolved), a deship Lie of bevreado new diversavob failed the stadmob saliartana sections (Fir. 5). This arrest offernall on bacane an orderly rational nevel offentions of the immelant and and eter optenliy. A herenel ordering of these calls was apparent. when victor block it that and to to that's methods with the second of th "The Tebial black of bone was very thin and demonstrated

perforating vasoriar chargels from the periodonici liseden: anasterosing with these of the labial periodress (Fig. 27-29). Major blood reseals were also observed in the mid-pertion of the lineal singles. In the periodresi area rasials:

channels of first caliber wars noted (216.2). Linear argumphilis structures appared to drightate in the mid-portion of the periodental lightent. This can be the intermediates places area or an area of fiber reorientation. From this area the fibers coursed towards the cemento-dental junction, where they terminated in an indistinct mass of neural and collagenous elements (Fig. 12). These fibers entered the cemental area and there branched and decusated to their terminus (Fig. 14). In the alveolar phase of the ligament bundles of collagen were seen in cross section surrounded by a limiting structure of darker staining quality (Fig. 20). In areas where fragments of these collagenous bundles remained due to incomplete digestion, it was observed that these limiting structures were neural elements which encompassed one or several Sharpey's bundles (Fig. 25,26). These looping configurations were not of equal diameter and exhibited wide variance from field to field.

The bundles of collagen originated in the periodontal ligament and followed a wavy course to alveolar bone where they inserted as Sharpey's fiber bundles. These bundles could be followed to the junction of the bundle bone and cancellous bone where they abruptly terminated (Fig. 30-32, 34,35). At higher magnification (Fig. 33) the tortuous nature of Sharpey's fiber bundles was easily discerned. It was observed that many of the deeply penetrating, incompletely digested Sharpey's fiber bundles demonstrated equidistant periodic banding across their width. These striations appeared to be related to the fibers of the collagenous matrix of bone which ran at right angles to the periosteal collagen bundles.

From this area the fibers coursed beamfe the commondered junction, where they terminated in an indistinct case of mergal and colladerous elements (Ele. 12). These fibers matered the communication area and there irreboked and denumbed to their terminus (Fig. 34). In the alveolar phase of the rounded by a limiting structure of deriver staining availably (Elg. 20). In areas where fragments of these collectors that these limiting structures were neural elements which that these limiting entrations were neural elements which associated and or every finiting the structures were neural elements which these looping configurations were neural elements which these looping configurations were neural elements and exhibited wide variance from field to field.

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Sharpey's fiber bundles were also demonstrable in the lingual alveolar bone where they originated in the periosteum.

On occasion, blood vessels of the periodontal ligament were seen to course to the gingiva where they anastomosed with vessels of this area. In sections through the col the confluence of ligament and gingival vessels was also apparent (Fig. 1,40,41).

# EXPERIMENTAL SECTIONS

Periodontal Ligament -- Alveolar Phase

In the alveolar phase of the coronal periodontal ligament of digested sections, large caliber vascular channels were found to course with thick nerve bundles (Fig. 13,23,24). The complex rete of blood vessels in this phase apparently developed an involved network of nerve fibers. As smaller diameter vessels branched to their terminus, neural filaments continued their intimate association (Fig. 23,24). This juxtaposition of structures was evident even to the capillary bed (Fig. 25). The characteristic pattern of nerve fibers in this area was a myriad of large coils and loops (Fig. 24,25). This anatomical configuration appeared to relate both to the capillary rete as well as to the collagen fiber bundles present in the ligament. As a result of the removal of the fiber apparatus by the collagenase, it was difficult to determine whether the coils or loops lay on the principal fiber bundles or twisted about them. However, in areas where

Sharpey's fiber bundles were also decomptuable in the lingual alveolar bune where they originated in the puriostaum.

On occasion, block rescale of the periodonial ligenood ware seen to course to the singiful where they analtoweed with vessels of this area. Is settion through the col the confluence of ligenant and singiful respets was also apparent (Fig. 1,40,41).

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insecold Laterbolizer Lecoros and to easis welcevia ent mi of divested sections, large caliber vascular channels ware (da, 23, 21, all) selfour serve bundles (Fig. 13, 23, 24). The courd at rete of blood versels to this where the base of developed as involved network of nerve fibers, is dealist diamain veges a branches to their versions, neurol filanesto continued their intinute association (Fig. 23, 24), This instanceiffice of structures was suidant even to the confilern hed, (Fig. 26). The characteristic pattern of norve fibers in this stee was a wright of large ootin and loops (Etc. 24, 25). add ed dtod edeled of harmente moltante to relate both to the cepillary reis as well as to the collegen fiber hundles present in the liveset. As a result of the removal of the fiber apparatus by the collassenage, it was difficult to determine whether the soils or loops lay on the principal close pundles of triated about them. However, is break where

remnants of Sharpey's fiber bundles were still in evidence (indicating incomplete digestion) nerve elements were seen to encircle areas previously occupied by the fibers (Fig. 24-26). This relationship suggests that there are nerve fibers that course around the collagen bundles. These nerves appeared to be fairly uniform in diameter, some gently tapering to their endings (Fig. 26). In addition, neural elements ending in the connective tissue appeared to have small nodular swellings along their length (Fig. 26) and terminated as free endings or with small swellings at their tips.

Vascular channels were observed perforating the cribriform plate of the alveolus from marrow spaces (Fig. 24). The relationship of nerve fibers to vessels and connective tissue in these areas was similar to that seen in the periosteal phase of the ligament. Organized corpuscular types of nerve endings were not seen although there was much evidence of free endings and knob-like structures.

Periodontal Ligament -- Cemental Phase

In the cemental phase of the periodontal ligament the nerve fibers coursed rather straightly from the mid-portion of the ligament, following the vasculature to the cementum (Fig. 13). Here neural elements were observed to branch and fork prior to entering the cemental area so that a more diffuse innervation was effected (Fig. 15,17,19). Near the tooth surface, terminal ramifications occurred with the disaggregation of nerve bundles creating a complex cemental

remnate of Sharowis fiber bundles were still in evidence (indicating incomplete disection) more electric were seen to ensirile creas previoualy occupied by the fibers (M.e. 20-26). This relationship supercisi that there are nerve fibers that course and the chies collegen bundles. There are nerve fibers poared toobs frinky entropy in diamber, some keeply tabenin to their endings (Fig. 20). In addition, neural elecents ending in the connective these appeared to have scale nodular availables along their fibers (S.e. 26) and tordies of as free and tool of the usefit ordifies of their tips.

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Periodontel Linement -- Centrobolie?

In the constant phase of the periodontal ligament the nerve dibers courded rather straightly from the add-portion of the ligement, following the varoulature of the constant (Fig. 13). Here nevel clearnts were observed to brinch and fork prior to entering the cenental area to bet a fore diffrage inservation are effected (Fig. 15,17,19). Near the tooth carface, terminal radifications occurred with the disneural plexus. Occasionally, the looping structures that characterized the alveolar phase were seen in this segment of the ligament. Varicosities were noted on the terminal nerve filaments. The nerve fibers in the mid-portion of the ligament were of larger caliber than those observed close to the cementum. It appeared, that with branching and decussation, their diameter decreased, and they terminated as fine fibers either at or within the cementum or the connective tissue (Fig. 16,18,20). As a result of the technique and the thickness of the sections, it is difficult to pinpoint the terminations of the nerves. Fibers were also observed to travel at right angles to the cemento-dental junction either within the cementum or on its surface for short distances (Fig. 17). In this cemental area, fusiform shaped bodies with short fibers emanating from them were sometimes seen. Fibers also ended in a smooth taper. Occasionally they were seen to enter the area of the cementum, swing back alveolarly and once again turn and terminate at or within the cementum (Fig. 18,20).

The majority of the fiber ends were of similar fine caliber. However, fibers of larger diameter were also viewed. These were not present in any orderly pattern but were randomly interspersed among the many finer fibrils.

This investigator was unable to observe complex endings of the corpuscular types that have been noted in other areas of the body.

nourel siere. Coosilonally, the looping structures tant. characterited the altient phase store seat in this sevent of the linement, where any time same noted on the terminal Itestatt wars of light wallber than then observed close to -version bein antrionered state thet , betersage the instrumentine tion, thats diameters discreted, and they berningted as Sind Riberd elther at an iting the centration or the compaction tizate (Fig. 16, 20). As a result of the technicks and the thickness of the sections, it is difficult to timestal the testimation of the samear. Filence wate of also observed to travel at winkt and de to the cenents is formed of the eithe mithin the cesentur of an its surfass for short dintances (Fic, 12). In this consists, area, furiford signal boiles with short fibers spansting from then were roustings sten. Phara also emittin in a sporth tabet. Sconstenelly tites terre sten to entres the area of the serentus, suiter back aistiv to the attentions and this sign has strafosvis the constitut (Mg. 28,20). will the maintain of the fiber outs asses offeted in fine califer. Rotever, fiters of larger dismeter were also there there were not present in any orderin but more mattingly intervenent anone the many fines fills, Shie investinator was maile to diserve complet with a

of the corresponder types that have been noted in other areas

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# Lingual Subepithelial Area

The lingual gingival epithelium was characterized by a keratinized surface, and was of undiscernable type due to the poor cellular detail inherent with the Gordon-Sweet Silver Stain. The definitive "deep plexus" of nerves as described by Dixon (10) was observed in these tissues. As the major vessels (in the supra-periosteal area) supplied the lamina propria, so did the larger nerves (Fig. 3-5). This relationship of nerves to blood vessels remained constant (Fig. 3). As the vessels ramified laterally forming the peripheral capillary bed of the gingiva, the nerve bundles divided and formed the familiar arborizing pattern of large loops and coils that were observed elsewhere in the periodontium (Fig. 3-5,10,11). Here, in the outer one-third of the gingiva, a definitive area was encountered which corresponded to the "superficial plexus." Subjacent to the basal layer of the epithelium were found curvi-linear and linear structures. Nerve fibers that appeared to circumscribe collagen fiber bundles seemed to be of larger caliber than those ending in the connective tissue. Fibers innervating the lamina propria appeared to have varicosities distributed along their length which were of apparent uniform width (Fig. 11). These coursed in relatively straight paths and did not appear to have a particular orientation to the epithelium (Fig. 11).

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Labial Gingiva

The major blood vessels to the labial marginal gingiva coursed in the medial portion of the connective tissue, sending branches to the labial, the sulcular, and the odontogenic epithelia. In both epithelial phases large looping neural structures were seen although this was more pronounced subjacent to the amelogenic cells (Fig. 8). Occasionally small singular loops were observed. Cigar shaped bodies, fine branched endings, as well as knob-like structures, were observed. At loo0x magnification no nerve fibers were observed to enter the overlying epithelium (Fig. 11).

Long epithelial ingrowths were consistently noted in areas that appeared to correspond to the by-pass area of the epithelial attachment. Apical to this area rete pegs were developed in an orderly arrangement in the ameloblastic phase. Superior to the by-pass area the sulcular epithelium exhibited few, shallow, or no rete peg formation (Fig. 6,7). In areas of greater cellular definition the labial epithelium demonstrated both a keratinized and parakeratinized surface. From the sections it was not possible to relate this variation in surface characteristics to changes in the underlying connective tissue.

Vascular Rete of Periodontal Ligament

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channels of two periodontal ligaments were observed (Fig. 39). Tracing these vessels recreated a large portion of two mesial periodontal ligament vascular retes (Fig. 40,41). Vessels of various diameters were traced both laterally and occlusally to anastomoses with vessels of the col and gingival areas.

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#### DISCUSSION

For the last fifteen years there has been an intensive interest in the identification and characterization of the proteolytic enzyme, collagenase. Studies have had as an objective the demonstration of the action of this compound in various physiologic and pathologic situations such as: the involuting uterus; the metamorphosing tadpole tail; and inflammatory states under the influence of normal and experimental dictates. Investigations have also been directed at the role collagenase plays in various dystrophic diseases. All this effort demonstrates that: collagenases can be produced exogenously or endogenously in relation to the tissue; collagenases from different sources effect different end products as a result of their varied mode of action; and the function of collagenase in the chain of collagen degradation is not fully understood.

Because of its specific action, several investigators have attempted to utilize collagenase as a histologic tool (68,70). This material has its greatest potential in areas where dense collagenation obscures the structures desired to be studied. This is precisely the situation in the periodontium if one wishes to discerne innervation. The oral anatomist is also confronted with the problem of decalcifying tissue and preserving its reactivity as a substrate for digestion. Green (68) reported that many of the common fixatives, such as Zenkers fluid, formalin, Bouins fluid, buffered osmic acid, buffered ocrolein, formalin-sublimate



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toteret in the Maniffrontion and characterization of the protective the demonstration of the action of this compound to review physiclesis and pathologic situations nuch as the involution where, the astamorphosine balance of inflamatory states under the influence of normal and experisons districts. Investigations have also been directed as the rolp officience plays in various Systemphic distance as the rolp officience plays in various Systemphic distance and as a reader of antophotal the rolation to the inclusion collagenases can be that, sollagenases can be mothe theory of antophotal that courses affect different and anotation of collagenase that and and of the theories the state at a reader of the information of notification is products as a reader of their varied ands of motion, and the traction of collagenase in a course of a collagenase can be the state of a collagenase in a collagenase of motion and the products as a reader of their varied ands of motion and the pothetic of a collagenase in a collagen defined bio traction of collagenase in a constant of collagen defined bio the pothetic of the state of a collagen defined bio

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for the lest fifteen years there has been an intensive

or Heidenhains susa, render collagen indigestible to collagenase, although Carnoy's ethanol-chloroform-acetic acid (6:3:1) or ethanol are found to be satisfactory. Mandl (66,67) observes that Hg++ completely and Ag++ to a lesser extent inhibit the enzyme as do most metalic ions in varying degrees. On the other hand Mg++, Mn++ and Co++ increase collagenase activity slightly and Ca++ does so twofold. Gallop (69) states that Ca++ is required in the substrate in both the suspended and solid phases; and that EDTA (Sodium Ethylenediamine Tetraacetic Acid) by its property of calcium chelation considerably diminishes collagenase action upon the substrate. Taking these facts into account, fixation with Carnoy's solution and decalcification with 5% Nitric acid is accomplished without deactivation of the collagenase. Vice (70) observes that digestion of unfixed sections results in unlimited destruction of the tissues and therefore predigestion fixation is desirable. While formalin inactivates the collagenase, she finds that its use after digestion enhances silver impregnation of the tissues. This investigator finds that post-digestion fixation is not essential since with the described technique effective staining of the argyrophilic elements can be achieved.

Collagenase preparations obtained from Clostridium histolyticum are often contaminated with other proteolytic enzymes. Ion-exchange chromatography has been suggested as a means of separating these unwanted enzymes from the clostridial extract (70). There is also a difference in

or Soldenhains sode, render colleres indicestible to oficeretorolic-levels styears? daueddie , saeresefice abid (6:3:1) on athanol are found to Ta real rectory. And) (56,67) otherwar that Hevet considerative and state to a leases enterer at another the entrue of an month metal to love in verying depress. In the offer hund Mart, Mart and Cott inortanes blotnet og good tend bin vittestiv ent does so tenfold. Gallen (59) states that Can la required in the addition in both the Paranalas and colld charges; and bigst Spin (Section Ribylessitenties welraateble Actal by Its processly of chickers obeletton considerelle ciministeres collerenere ertica voon the hubstrate. Saline these facts into socount, fixation with Carport's solation and decalor floation with 5% Micris adid in socomplithed without deaptiestion of the collegeness. Vica (26) observer that digestion of unfixed weetlorn results in welterted destruction of the tingues and therefore predices tion freiter is desirable. While formalin fractivets, the collegename, she finds that its use after disention entenness shill totestize the tis tis tis and . This investigator finds that root-distantion firstion is not assemblal since with the described beinters affective statement of the argumentics . Dave id the so man be so his state

Collagendre provaretions obtained from Clostridium hidteletter are often contaminated with other protoclytic enzymes. Ich-dichange chromotography has been anglested an a means of separating these unwanted enzymes from the clostridial extract (70). There is also a difference in

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collagenase concentration between enzyme lots and for this reason 200ml aliquots of digesting solution were prepared at one time. Compounding the solution in this fashion minimizes variation in tissue hydrolysis and constant time and temperature factors can be maintained.

Investigators have classically employed various techniques to suppress the staining qualities of collagen and reticulin. The literature of neurohistology is replete with modifications developed to accomplish this end. Bernick in 1955 (1) reports that treatment of tissue sections with pepsin or trypsin, while not completely removing collagenous fibers, allows him to identify and characterize the nerve supply to the teeth and periodontal structures.

Collagenase achieves removal of unwanted elements and permits an unobstructed view of neural structures. Time and temperature of the collagenase bath are critical for optimal hydrolysis of collagenous structures. If these are not carefully monitored, the sections undergoing treatment are macerated to the extent that they are undecipherable. At the specified concentration, time, and temperature employed,  $(5mg/ml \text{ for } 33 \text{ minutes at } 45^{\circ}\text{C})$ , all collagen including that which comprises the organic matrix of bone and tooth dissolves so that experimental tissues totally lack these structures, i.e. sections through the pulpal tissues appear to be suspended in space. Only by imagining the presence of dentin can the pulp tissue be related to the total section. In those areas where a continuation of periodontal soft

collegences concertration between enzyme lots tof this reason 200ml aftquate of digesting solution warp prepared at one time. Compounding the solution in this fashion ministees variables in tissue hydrolysis and constant time and temporation forther can be saintained.

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Collectors with ever removal of unrember allowed and presite on maintrupter view of nounal structures. If and temperature of the collectors both are existent for optimal buddeleds of collectors structures. If there are not such that remitered, the restions universing treatment are relify conitored, the restions universing treatment are associated to the extent thet stop are undecipatentia. At the sociaties concentration, these and temperature scales, (Star's) for 33 simples at  $10^{\circ}$ ), all collears to bar which complete the extent tips were totally have the structures, i.e. sections through the rules) issued appear attractures, i.e. sections through the rules) issued appear dentic can the quip tienes be related to the boil section to be the superime be related to the boil section. tissue exists from buccal to lingual via the periodontal ligament, it appears as a hammock suspended between two peaks. This configuration is due to the orientation of the incisor to the saggital plane which results in sections being obtained that are tangential to the long axis of the tooth.

The peripheral branches of the gingival nerves create an extensive plexus in the subepithelial zone. This neural rete, partially sympathetic and partially afferent, is associated with the corresponding subepithelial vascular plexus of arterioles, venules and capillaries. The gingival nerves include both the afferent fibers which terminate in this area as well as the sympathetic fibers through which the gingival blood vessels receive their efferent innervation. The afferent supply consists of both myelinated and amyelinated fibers; the sympathetic are mainly amyelinated.

This investigation unsuccessfully attempted to stain for myelin with Luxol Fast Blue stain after treatment with collagenase. As part of the preparation for staining, the slides were colloidinized, to maintain juxtaposition of parts, and it is probable that the ethyl ether-absolute alcohol bath removed the myelin fraction from the nerves. Additional studies are presently underway with modifications in the handling of the tissue sections so that staining may disclose the ramification and relationships of myelinated and amyelinated fibers.


theme exists from buscal to inequal via the periodontal ligement, it appears at a metadok samp moded beganes into peaks. This configuration is due to the prisemation of the incirct to the segmital place which results in sections being obtained that are targenized by the lone arts of the tooth.

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While investignation moreoresefully attempted to stain for symplex with lined last Alus stain after freetants with collegeness, is put of the preparation for staining, the slides were colloidinized, to subtain justeposition of nerts, and it is probable that the structure from the statemalcohol bath removed the sublik fraction from the notifications additional similar or investign fraction of the test and the statement of the statementation from the notifications disclose the test fibers of the statement of the statement the statement of the statement scatter and the statement additional similar of the statement statement of the disclose the test fibers, and the statement of the statement disclose the test fibers, and the statement of the statement of a statement of the statement statement of the disclose the test fibers, in the statement of t

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The subepithelial or "superficial plexus" (13) forming in both labial and lingual gingivae is due to the arcuating configuration of nerves coursing with the terminal vascular bed. These nerves form circular structures which are so oriented as to lie upon the length, or around the circumference of, the collagen fiber bundles. In areas where epithelial rete pegs are prominent some of the nerve fibers associated with the vasculature terminate in relation to the vessels while others branch to join the subepithelial arborizing plexus. This is seen in areas where rete pegs are not pronounced as well as in the alveolar phase of the periodontal ligament.

The periodontal innervation is composed of relatively slender fibers which vary in caliber but do not present distinct classes according to size. Nerves that end in complex structures are reportedly medium or small diameter fibers (32). Since lamellated organs were not observed in the periodontium it consequently was not possible to correlate nerve fiber diameter to terminus type and theoretical function. Some of the straighter nerve bundles, originating in the deeper tissues vary in diameter, but all terminal loops are approximately the same size. It is possible to speculate that all sensation is mediated through similar diameter terminal nerve fibers.

This study substantiates Bernick's findings (2,4,8) that complex end organs may not be present in the lamina propria of the periodontal apparatus. The fact that complex



The subspheredial of "superficted planes" (1) former in both lestel and limital singles is at to the erotating configuration of nerves rootsing with the bornhal capable bed. These serves form sirrocher structures which are to orighted as to lie and the length, or around the sirenformers of, the collecte fiber buddles. In areas where collected with the cape are produced come of the nerve fiber secondated with the cape are produced come of the nerve fiber rescale while offers tranch to join the succeduality arborising description to be allow the second back of are not promoved as will be in the electronic to be are not promoved as will be in the electronic best for are not promoved as will be the second back of the second back of are not promoved as will be an in the electronic best of the are not promoved as will be an in the electronic best of are not promoved as will be the second back of the second back of are not promoved as will be an in the electronic best of are not promoved as will be an in the electronic best of are not promoved as will be an in the electronic best of contained fiberents.

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This staty exclationtiates Servicies findings (2, 2, 8) that complete and organs may not be present in the baning progris of the periodonial apparetus. The fact that coupler

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end organs are not observed in studies employing enzymatic removal of collagen and reticulin does not necessarily prove nor disprove that these neural elements are present in these tissues. It is quite possible that the fine end body capsules and lamellated structures are so severely affected by enzyme dissolution that they are totally destroyed and this might lead one to erroneously assume that they do not exist. Varicosities along the length of nerve filaments are still identifiable as well as terminal arborizations which occasionally are seen to end in knob-like or expanded tip structures.

A consequence of the tangential plane incisor section is a view in which the periodontal ligament is bounded by: the tooth superiorly; the alveolar bone inferiorly; and the labial and lingual gingivae laterally. In studying these sections it can be seen that in the mid-portion of the ligament there is a change in fiber orientation from horizontal in the alveolar phase to vertical in the cemental phase. This zone of directional transition appears to correspond to the intermediate plexus described by Goldman (51). Not only are the collagen fibers observed creating this pattern but the neural elements also play a role. In digested slides linear nerve tracts are viewed in the cemental aspect of the ligament while arborizing figures are characteristic in the bony side of the ligament. If in the cemental phase nerve tracts coiled about bundles of principal fibers then spiral nerve tracts would be seen in this zone after sectioning



and organs are not observed in similar employing enzymetic removed of solitares and coticulto does not necessarily prove nor disperve that these neural disports are present in these bilarys. It is quite constills that the fine and body and calcered issellated structures are so severaly affected by anyon these districtions that body are so severaly affected by where there insi there are totally destroyed and this is affected in that body are totally destroyed and this distribution the issues of areas being the the origin that body the second of areas of a several and this second these along the length of arrow filtenets are still identifiable as well as torsimal arborizations which are still and the second of a body and the second of the second time of a second of a body are the origin the still be a born and a storigation of the origin enterty of the second of a body are the origin.

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the tissues. Only a few such structures are to be found. By the same token, straight tracts in the alveolar zone, coursing parallel to the long axis of principal fibers, and cut in cross section, would appear as many argyrophilic dots. The explanation for this difference in fiber orientation is unclear and additional studies are contemplated to compare this configuration about other teeth from the same and other species.

Straight nerve tracts in the cemental phase are sometimes observed to have arborizing lateral branches giving innervation to adjacent connective tissue. The linear fibers appear to be oriented so as to terminate head-on at the cemental surface. This relationship with the tooth may be a function of the physiology of the constantly erupting incisor. As a consequence of continual eruption and chronic dentin erosion, Hattyasy (71) has projected that the pulp lacks sensory innervation. It is his belief that these teeth would be extremely sensitive if they had such nerve supply. It is also possible that the cemental surface of the rat tooth may not contain fixed innervation. Bernick (72) has suggested that nerve fibers inhibit calcification of their extremities. If this is the case then the periodontal ligament nerves of the constantly erupting incisors stay fairly fixed in position and in effect are sliding across the cemental surface as it exfoliates.

In several wholly or partially undigested sections the insertion and depth of penetration of Sharpey's Fibers in



the trades. Loty of a store the structures are to be forch. By the rate take, straight tradet in the alvelur sens, contains paralied to the long aris of arthoural fibers, and out in error section, whill expense a take the tradet lie doin, the section attact of the difference in fiber origination to used are and additioned attacted are donicated to another this conditates for about ather sections to contain the this conditates about ather section from the same and other spotes.

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In several whelly or partially undigested sections the trace the finance the finance the second and depth of perstration of Sharpay's Pibers in

bone are distinctly stained. It is observed that they penetrate the buccal or lingual alveolar bone only to the bundle bone-cancellous bone interface. At this junction they terminate abruptly. This seems to confirm Provenza's contention (54) that they are of periosteal origin rather than a product of continuous fibroblastic secretion during the development of the jaws.

In all areas studied, nerves opposit to and in knot-lis structures or free andings. Consist and organ configuration are not seen but this may be a result of the sollareness discertion of the tissner.

Sharpey's Fibers are observed inserting into the print of the case and releved a set be stall are interface.

Blood massis are charved anastomoving between the second light of the basis of the second light for the second light of the se



bone are distinction statistics. It is observed that they prostrate the model or lingual alvealer bone only to the build a pose-dencellous bone interface. At this junction they terminate attraction while seems to confirm Provense's contention (C4) that they are of periodesi origin rather than a product of confirmance firmblictic secretion during the development of the junct.

### SUMMARY

A method has been described whereby tissues containing ossified components are decalcified and subjected to enzymatic digestion with collagenase.

The labial and lingual gingivae of the constantly erupting mandibular incisors of the Sprague-Dawley rat are seen to be well innervated. Arcuating nerve fibers forming a discrete superficial plexus are observed.

Nerve fiber tracts encircling principal collagen fiber bundles are characteristic of the alveolar phase of the periodontal ligament. In the cemental phase nerves course fairly straight to their terminus from a zone in the midportion of the ligament which corresponds to the "Intermediate Plexus" described by Goldman.

In all areas studied, nerves appear to end in knob-like structures or free endings. Complex end organ configurations are not seen but this may be a result of the collagenase digestion of the tissues.

Sharpey's Fibers are observed inserting into the cribriform plate of the alveolar bone where they abruptly terminate at the cancellous bone interface.

Blood vessels are observed anastomosing between the periodontal ligament, col, and labial or lingual gingiva.



### THANHUS.

A nethod was been described whereby those containts osaified compose and decale field and and contains to entry astic direction with collarenase.

The lottel and limited singly and of the constantly erroting meditionar incloses of the Spramo-Dawley rot are seen to be well inversated. Spraming sorre fibers forsing a discrete supplied flexus are observed.

Herve fiber tracks excitating principal colored fiber bundles and contrateristic of the sivelar phase of the periodental lighters. In the communic phase herves course fairly attribute to their terminus from a core in the sidperfice of the lighters which corresponds to the "Internetiate Element described by folders.

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Unarray's Hhere are cheered intering into the orthorized into the orthorized of the alvesiar bone where they ebruntly terminate of the careellous bone interface.

stood vessels are observed anartomosing between the period present, out, and labial or lineas findiva.

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#### 12.

## APPENDIX A

Modified Gordon-Sweet Silver Stain Procedure

- 1. Oxidize in acidified permanganate for 2 minutes
- 2. Wash in distilled water for 1-1 minute
- 3. Bleach in 1% oxalic acid for 1 minute
- 4. Wash well in 3 changes of glass distilled water
- 5. Mordant in 2% Iron Alum for 72 minutes
- 6. Wash in 3 changes of glass distilled water
- 7. Impregnate in Wilders Silver bath for 20 seconds
- 8. Rinse well in distilled water
- 9. Reduce in 10% neutral formalin for  $\frac{1}{2}$ -1 minute
- 10. Rinse in 95% alcohol and dehydrate in 100% alcohol
- 11. Rinse in Xylol and mount with permount



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# A RIGARIA

Rodlfied	Gordon-Sweet Silver Staln Frederice
.1	estette te actdified purcangamate for 2 minutes
.5	wants in distilled weber for get alloute
10	Bleach in 15 challe sold for 1 minute
	vetor follitals saving to seaded 5 ml liev need
· Č	Hordent in 25 Iron Alus for 24 minutes
.3 1.1	Wash in 3 binnees of plais distilled water
7.	Ispresses in Wilders Bilver bach for 20 seamls
.8 <sup>8.364</sup>	Three well in Stabilled water
.0	Reduce in 16% neutral formalis for 4-1 minute
10.	Rinse in 99% alcohol and demystrate in 100% alcohol
.11	Ringe in 19101 and mount with permount

33 APPENDIX B

# Phosphate buffer solution

M/15	Na2HPO4	400
M/15	KH2PO4	lcc
0.9%	NaCl	500

## APPENDIX C

# Collagenase Digestion Solution

Collagenase			5mg
Phosphate	buffer	solution	lcc





## APPENDIX D

## Key to Plate Abbreviations

- a artifact
- ae amelogenic epithelium
- af argyrophilic fibers of PDL
- afx argyrophilic fibers of PDL in x-section
- ag argyrophilic fibers of gingiva
- b bone
- bb bundle bone
- BN branching nerve fiber
- br basal reticulin layer
- c cementum
- cb cancellous bone
- CN coiled nerve tracts
- d dentin
- e epithelium
- eb epithelial bypass
- es enamel space
- f principal fibers of PDL
- K end knobs
- kf Korff's fiber
- la labial
- le labial epithelium
- LN large nerve coils
- m muscle bundles
- NS nerves around Sharpey's Fiber remnants



## APPENDIX D

Enclasiverida staff of tel

- antifact i
- as anelogenic solthelium
- af anyrophillo filers of ED
- afx sreyrochills fibers of FDL in m-section
  - avianta to credit of indercare as
    - b bone
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    - modifi evicon anidomand MB
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- Ef Eorff's filter
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- NV neural varicosity
- o odontoblastic layer
- p pulp
- pl periodontal ligament
- pr perforating vascular channel
- s Sharpey's Fibers
- se sulcular epithelium
- SN straight nerve tracts
- tc terminal capillary bed
- TN tight nerve coils
- TNE terminal nerve endings
- v vascular channels
- VN nerves passing along vascular channels
- vr vascular reticulin
- z zone of fiber orientation transition



- vilacoliny ferues VH
- a dontoblastic layer
  - a lua a
- tromanil introdetter . In
- pr perforating vascular channel
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Fig. 1 Control section of lingual gingiva and periodontal ligament. Epithelium (e) appears golden to brown while the dentin (d) and lingual alveolar bone (b) are rust colored. The fiber apparatus of the periodontal ligament (pl) and gingiva is dark brown. Observe a blood vessel (arrows) coursing occlusally to anastomose with vessels of the gingiva here seen in cross section. Gordon-Sweet Silver Stain. Mag 100x

Fig. 2 Control section of lingual attached gingiva. Note that although the epithelium (e) stains darkly, the basal cell area exhibits a lighter coloration. In the area of the basement membrane, the deep staining subepithelial reticulin layer (br) can be found. Coursing midway between the epithelium and bone (b) a large vascular channel (v) is observed with its circumferential reticulin layer (vr). Smaller vascular channels may also be viewed cut at varying angles to their trajectories. With the staining of the dense collagenous and reticulin network, one cannot define neural elements present. Mag 150x





Fig.2







Fig. 3,4,5 Serial apico-occlusal sections of lingual gingiva after enzymatic digestion. These photomicrographs of an area similar to Fig. 2 demonstrate a large gingival vessel with its vascular nerves (VN) coursing parallel to its long axis. The superficial plexus of large looping neural structures (LN) are quite evident as the non-neural argyrophilic fibers have been removed by the collagenase. There are however several areas where the subepithelial reticulin fiber system is still present. Mag 100x







Fig. 6 Control section of labial gingiva. A prominent downgrowing epithelial rete peg (eb) with several secondary projections is observed arising from the area of the epithelial attachment. The sulcular epithelium (se), labial epithelium (le) and amelogenic epithelium (ae) are of fairly uniform dimensions. There is a suggestion of arborizing argyrophilic fibers (ag) in the lamina propria; no differentiation can be made as to the nature of the structures creating this pattern. An artifact of an enamel space (es) has been created by decalcification with nitric acid. Mag 100x

Treated section of labial gingiva. In this Fig. 7 experimental section of an area similar to Fig. 6, surface relationships are the same -- although in mirror image -- but now through enzymatic dissection the neural rete of the connective tissue is clearly evident. Large coiling nerve filaments (LN) can be seen subjacent to the amelogenic and sulcular epithelia while beneath the labial epithelium this configuration, although present, is not as extensive. A large vascular channel (v) can be seen in the mid-portion of the lamina propria sending branches laterally as it courses to the free gingiva. The deeply penetrating epithelial bypass is prominent and appears to be at the coronal limit of amelogenic rete peg formation. Mag 100x

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Fig. 8 Arborizing neural structures and terminal endings in the subameloblastic zone. Subjacent to the odontogenic epithelium (ae) knob-like terminal endings (K) and smaller looping structures (TN) as well as larger coiled filaments (LN) can be found. Near the bottom of the photomicrograph, a portion of a deeply penetrating epithelial rete peg (eb) from the by-pass area is observed. Fragments of the subepithelial reticulin can be noted adjacent to the ameloblestic basal cells. Mag 430x

48







Fig. 9 Knob-like end organs in the dermal papillae. Expanded tip terminal structures (arrows) are viewed in this photomicrograph of the amelogenic epithelium dermal papilla. The right pair of end organs appear to arise from a bifurcating looping nerve fiber. The numerous small punctate bodies along the periphery of the papillae are residual elements of the reticulin rete. Mag 1000x

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Fig. 10 Lingual fornix -- experimental section. Superficial nerve plexus of the lingual fornix is viewed here as large circling structures (LN). A vascular channel (v) can be observed coursing vertically towards the epithelium (e). Mag 430x

Fig. 11 Oil immersion of Fig. 10 insert. Coiled terminal arborizations (CN) and neural varicosities (NV) are visible. Note the nerve fibers in the subepithelial zone terminating (TNE) in the connective tissue beneath the epithelium (e). Mag 1000x

52











Fig. 12 Control section of the coronal periodontal ligament. Blood vessels (v) are observed to occupy a major portion of the alveolar phase. Here argyrophilic fibers cut in cross section (afx) can be found interspersed between the vascular channels. Midway between the crestal alveolar bone (b) and the dentin (d) there is a change in argyrophilic fiber (af) orientation creating a readily identifiable zone. In the cemental phase of the periodontal ligament the fibers course fairly straight to their insertion into the cementum (c). Mag 100x

Fig. 13 Enzymatic digestion of coronal periodontal ligament. Due to the removal of nonnervous argyrophilic fiber by collagenase hydrolysis, vascular nerves (VN) as well as nerves coursing directly towards the cementum (SN) are clearly evident. In the alveolar phase nerves that coiled about Transseptal or Group C fibers (CN) can be seen. Mag 110x







Fig. 14 Control section of cemental phase of coronal PDL. Linear argyrophilic fibers (af) are viewed coursing to the cementum (c) in this undigested section. Collagen and reticulin element mask the neural elements. Mag 400x

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Fig. 15 Cemental phase of coronal PDL after digestion. The linear nerve tracts (SN) are observed in this section. Note the lateral branching which are terminating in unexpanded tips. At the bottom of the plate can be seen the directional transition zone (z). Mag 430x

Fig. 16 Insert of Fig. 15. This photomicrograph demonstrates bifurcating nerve fibers (BN) and terminal nerve endings (TNE). Observe the fairly uniform diameter of the arborizing end branches. Mag 1000x



Fig.16

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Fig. 17 Cemental phase of coronal PDL after digestion. Straight nerve fibers (SN) coursing to their insertion in the cementum (c). Note the absence of lateral branching of the fibers except at their terminus. Mag 430x

Fig. 18 Insert of Fig. 17. Oil immersion photomicrograph in which a branching nerve fiber (BN) and terminal neural elements can be observed to insert into the cementum (c). The larger nerves deccusate into fibers of smaller diameter of uniform dimension. Mag 1000x

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Cemental phase of coronal PDL after digestion. Fig. 19 Cemental phase of periodontal ligament in which nerve fibers course from the directional transitional zone (z) to the cementum (c). A vascular channel (v) can also be observed travelling towards the cementum in a trajectory parallel to the nerve fibers. Mag 430x

Fig. 20 Cemental phase of coronal FDL after "ase." Oil immersion view of the cemental phase of the periodontal ligament. Here we can note nerve fibers branching to their terminus (BN) at the cementum (c). These neural elements appear of uniform diameter even to their ending (TNE). A fiber is also seen to enter the cementum, course towards the periodontal ligament and then return to the cementum. Mag 1000x

62

Fig.20




Fig. 21 Alveolar phase of PDL -- Control section. Control section of alveolar phase of coronal periododontal ligament in which one observes argyrophilic fibers (af) both in longitudinal and cross section. A fine fibrillar structure (N) can be noted in the lower right portion of the photomicrograph which has characteristic varicosities (NV) associated with neural elements. Mag 400x

Fig. 22 Alveolar phase of PDL -- Control section stained with Goldman-Bloom Trichrome Stain. A control section of the same general area as the preceding figure but treated with the Goldman-Bloom trichrome. Here the bone (b) appears crange and the fiber apparatus of the periodontal ligament, blue. Vascular channels (v) are also very evident in this section coursing parallel to the alveolar surface sending branches laterally to the ligament. Mag 110x







Fig. 23 Vascular channel and branching terminal nerves of alveolar phase. Oil immersion photomicrograph of a blood vessel in the alveolar phase of the periodontal ligament. Note the vascular nerves (VN) which course with the vessel as well as a branching nerve fiber (BN) forming coiled nerve structures (CN) and terminating as simple endings (TNE) in the connective tissue. Mag 1000x

Fig. 24 Perforating blood vessel and nerves from marrow space to PDL. A blood vessel with attendant vascular nerves (VN) is seen perforating the alveolar bone (b) from a marrow space to the periodontal ligament. Several intact, and remnants of, Sharpey's Fibers are seen in cross section. As vascular nerves branch from the vessel, coiled neural structures (CN) are created. Mag 400x









Fig. 25 Alveolar phase of deeper PDL after digestion. Experimental section through the alveolar phase of the periodontal ligament in which one notes the many coiled neural structures (CN). The terminal capillary bed (tc) is also evident as pale yellow tracts. In the upper right-hand corner alvoelar bone (b) with Sharpey Fibers cut in cross section can be seen. In addition several nerve fibers (NS) are seen to encircle areas of remnants of these principal fiber groups. Mag 430x

Fig. 26 Nerves coiling about Sharpey's Fibers. In this photomicrograph of an area of incomplete collagen digestion nerves (NS) are seen to scribe a course around remnants of Sharpey's Fibers (s). Where complete digestion has occured a void exists and a coiled neural structure (CN) is now apparent. Several nerves with varicosities (NV) are also visible. The terminal capillary bed (tc) can also be identified. Mag 1000x



Fig.25



Fig.26







Fig. 27 Vascular channel perforating labial plate -control section. A perforating vascular channel (pv) can be observed between two segments of the labial plate of bone (b) as outlined by the arrows. To the left can be observed the enamel space (es), the amelogenic epithelium (ae) and the periodontal ligament (pl). A portion of the alveolar plate has fractured during processing causing an artifact (a) which resembles a vascular channel (v). Mag 100x

Fig. 28 Vascular channel perforating labial plate -partial digestion. In this section vessels and attendant nerves can be viewed traveling from the periodontal ligament (pl) to the labial periosteum between segments of the alveolar plate of bone (b). A large vascular channel is viewed in the ligament. Mag 100x

Fig. 29 Vascular channel perforating labial plate --"ase." Blood vessels and nerves anastomosing with structures of the periodontal ligament and the labial periosteum. Mag 200x

70















Fig. 30 Control section demonstrating Sharpey's Fiber insertion. In this section Sharpey's Fibers (s) are seen to enter the bundle bone (bb) and stop abruptly at the junction with the cancellous bone (cb). Mag 100x

Fig. 31 Control section of Sharpey's Fibers insertion. In this photomicrograph fiber bundles are seen to originate in the periodontal ligament (pl) and to insert into the bundle bone (bb) of the mandible. A distinct transition occurs between bundle and cancellous bone in that no fiber bundles are observed in the latter. Several vascular channels (v) are observed partially partitioning this bone. Mag 150x

Fig. 32 Control section of Sharpey's Fiber insertion. Note the abrupt termination of fibers at the junction of the bundle and cancellous bone. Mag 200x









Fig. 33 Control section of alveolar bone surface. The wavy nature of Sharpey's Fibers are apparent in this section as they insert into the bundle bone (bb) from the periodontal ligament. Note the vascular channel coursing close to the bone surface. Mag 430x

Fig. 34 Partial digestion demonstrating Sharpey's Fibers insertion. This plate depicts the depth of penetration of principal fiber bundles (s) into the bone from the periodontal ligament (pl). Major vascular channels (v) are seen to lie in the alveolar phase of the ligament. Mag 100x

Fig 35 Sharpey's Fibers -- Partial digestion. Here the fibers are seen to have several curves of high amplitude in the ligament (pl) while in their insertion and after some digestion the amplitude is decreased although the frequency per unit length is increased. Mag 430x











Fig. 36 Korff's Fiber -- control. Arising near the periphery of the odontoblastic zone of the pulp is a tridentate structure defined as a Korff's fiber (kf). In the supra-odontoblastic area one can observe several blood vessels cut in cross section. Medially a large pulpal vascular channel (v) is seen. Mag 430x

Fig. 37 Korff's Fiber -- control. In this photomicrograph a Korff's fiber (kf) is seen to arise in the odontoblastic zone (o) of the pulp and extends into the dentin (d) for a considerable distance (arrows). Note the branched origin of this dense collagen bundle. Mag 430x

Fig. 38 Korff's Fiber -- partial digestion. In this experimental section several purplish staining collagenous fibers are viewed arising from the pulp (p) and penetrating the area of the dentin (d). Mag 600x

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Fig.36



Fig.38





Fig. 39 Blood vessels of the periodontal ligament. A tangential section through the PDL demonstrating a portion of the vascular rete found in the alveolar phase. Mag 60x



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Fig. 40 Graphic reconstruction of the vascular rete of the PDL. This plate was drawn from 25 serial sections of which fig.39 is an example. The alveolar bone (b) has been removed by the digestion process. Various caliber blood vessels of the ligament are evident and can be observed anastomosing with each other, and with vessels of the col and lingual gingiva. A large lingual gingival vessel (left arrow) may also be seen. The epithelium (e) is continuous over the col from labial to lingual. Mag 60x







Fig. 41 Graphic reconstruction of the vascular rete of the PDL. This plate was drawn from 30 serial sections. Various diameter vessels may be observed. Anastomoses with col and gingival vessels are visualized. The alveolar bone (b) has been removed during the treatment with the collagenase. The epithelium is continuous from labial to lingual over the col area. A portion of the labial musculature can still be seen. Mag 60x





