# A STUDY OF PERIODONTAL LIGAMENT MESIAL TO THE MOUSE MANDIBULAR FIRST MOLAR

A project submitted in partial fulfilment
of the requirements for the degree of
Master of Dental Surgery

by

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# SUMMARY

Eight, thirty five day old A.L.C.A. strain mice were anaesthetized and perfused with a cacodylate buffered glutaraldehyde and osmium tetroxide solution. The first molar was dissected from the mandible and prepared for TEM examination. Two animals comprised a pilot study. The ligament mesiobuccal to the mesial root was sectioned parallel to the occlusal plane from the alveolar crest to the tooth apex, and trisected into tooth, middle and bone thirds.

Each region was quantified using standard point counting procedures, and the data analysed using a general linear model. From these results it was determined that quantification of ultrathin sections, 200 microns apart, in three animals, was sufficient for analyses in the final study. Main effects due to animal, side, depth and zone across the ligament were examined and it was found that the only statistically significant variations occurred between different lateral thirds of the ligament.

The association of different structures was assessed by means of a Pearson correlation matrix. Statistically significant correlations in the number of profiles per micrograph included those between:

(i) myelinated and nonmyelinated nerves in blood vessel walls;

(ii) oxytalan fibres situated in blood vessel walls and postcapillary-sized vessels lacking pericytic cellular investment; and,

(iii) unmyelinated nerves not located in blood vessel walls and K-cells.

Volumetric proportion was calculated for all periodontal structures. Quantitatively, the tooth third of the ligament was the most cellular, the middle third contained the most oxytalan fibres and the bone third contained the greatest neural and vascular volumes.

Stereological parameters were calculated for blood vessels, nerve axons and oxytalan fibres. Of the periodontal blood volume, 88% was enclosed in vessels with a mean lumenal diameter of 20.9 microns which were characterized by a thin endothelial lining and few, if any, perivascular cells. The length density of the nerve axons within the ligament was 255.9 x $10^3$cm/cm$^3$. Unmyelinated axons, which constituted 95% the periodontal nerve fibres, had a mean caliper diameter of 1.4 microns. Oxytalan fibres had a length density of 1258 x $10^3$cm/cm$^3$ and a mean caliper diameter of 0.7 microns. Furthermore, 78% of fibres were present adjacent to cells, 14% within principal collagen fibres and 8% occurred in the walls of blood vessels.

These parameters demonstrate that the periodontal ligament is structurally different to other connective tissues for which stereological analyses have been undertaken. Because this tissue does not have the structure of a true ligament, it probably does not function as one. It would seem appropriate from this study that the generally accepted nomenclature of "periodontal ligament" be replaced by the term "periodontal attachment", as the latter infers tissue function but bears no misleading connotations relating to tissue structure.

## SIGNED STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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# CHAPTER 1

## INTRODUCTION

Periodontal ligament is the term most commonly used in reference to the soft connective tissue providing continuity between tooth cementum and alveolar bone. Although this tissue is not a typical ligament in either structure or function, other names such as periodontal membrane, pericementum and gomphosis are probably less appropriate (Melcher, 1976).

Functionally, the periodontal ligament provides the mechanism for eruption and tooth support. It is responsible for resisting functional and parafunctional displacing forces applied to teeth, and the recovery of tooth position when such forces are removed. Furthermore, the cells of the periodontal ligament not only maintain the ligament itself, but are also implicated in the resorption and repair of adjacent alveolar bone and cementum. Sensory receptors in the periodontal ligament appear to have an important proprioceptive function and may play a role in the control of masticatory movements.

Subjective, light microscopic description of this tissue has been undertaken for a century or more and, with the advent of the TEM, more detailed ultrastructural investigation has been possible. As materials used with this technique are expensive and because of the difficulty in obtaining adequately preserved human tissue, researchers have generally utilized rodent periodontal ligament for TEM studies. Such investigations usually consist of ultrastructural examination of

specific components, with fibroblasts and blood vessels being most commonly studied. Although morphological description is necessary, it has led to a limited understanding of structural organization within the periodontal ligament.

Scanning electron microscopic techniques, whilst providing excellent visualization of blood vessel corrosion castings, have limitations of tissue distortion and problems in determining the juxtaposition and organization of different components within the ligament. Similarly, light microscopic techniques have limitations of tissue shrinkage, microscope resolution and section thickness that make them less accurate for stereological investigation than TEM analyses (Weibel, 1979).

From the variety of techniques used to investigate this tissue, it is apparent that the distribution and orientation of periodontal structures is not uniform or random within the ligament (Carranza, Itoiz, Cabrini and Dotto, 1966; Kubota and Osanai, 1977; Scott and Symons, 1982; Berkovitz and Shore, 1982). However, most reports have limited statistical substantiation or have been based on subjective interpretation.

Numerous morphometric investigations of oral tissues, other than periodontal ligament have been undertaken, using light microscopic and TEM techniques. These include studies by Schroeder, Münzel-Pedrazzoli and Page (1973), Landay and Schroeder (1977) and Franklin and Craig (1978). Information obtained from these investigations has enabled objective comparison of changes occurring with age, function and disease.

Stereological analyses, describing three dimensional periodontal structure from ultrathin sections, have not been published to date. Although estimation of the volumetric proportions of various components has been undertaken for periodontal cells by Beertsen and Everts (1977), blood vessels by McCulloch and Melcher (1983), collagen by Shore and Berkovitz (1979) and oxytalan fibres by Jonas and Riede (1980), detailed morphometric analysis of the periodontal ligament has been lacking.

The mechanism of tooth support, physiological changes occurring with age and periodontal responses to parafunction, orthodontic tooth movement, trauma, infection, transplantation and neoplasia are not clearly understood. Stereological analysis of normal tissue is required so that tissue function, physiology and pathological periodontal changes can be assessed.

The present study was undertaken to examine the ultrastructure of profiles within the mouse molar periodontal ligament and to derive morphometric and stereological data describing the ligament mesial to the mouse molar.

A review of the literature has been included to indicate the criteria used for profile identification and to describe present concepts relating to distribution and volumetric proportion of structures that constitute the periodontal ligament.

# CHAPTER 2

## AIMS OF THE INVESTIGATION

The purpose of this study is to provide morphometric data for structures within the ligament mesial to the mouse molar. Consequently, the aims of the investigation were:

1. To prepare material for TEM examination of the mouse molar periodontal ligament.

2. To formulate a TEM study enabling the morphometric analysis of periodontal ligament mesial to the mouse molar to be undertaken.

3. To conduct a TEM examination of this tissue, in order to identify the cellular, vascular and fibrous structures present.

4. To derive morphometric data for the profiles investigated.

5. To determine statistical correlations of different structures within the ligament.

6. To calculate three dimensional stereological parameters for periodontal nerves, oxytalan fibres and blood vessels located mesial to the mouse molar.

# CHAPTER 3

## REVIEW OF THE LITERATURE

The periodontal ligament is the fibrous connective tissue located between the cemental surface of the tooth root and the periodontal surface of alveolar bone. The most coronal portion of the ligament is marked by fibres that extend from cementum to the alveolar crest of adjacent bone. This tissue is not a typical ligament in structure nor function (Melcher, 1976) and consequently has been the source of extensive investigation in an attempt to eludicate, not only tissue structure, but the method by which the tooth is supported. The microscopic anatomy of the periodontal ligament has been explored using a variety of animals and a number of laboratory techniques. Cohn (1957) and Atkinson (1972) state that the mouse molar periodontal ligament is similar in appearance and arrangement to the human ligament, and that results derived from investigations using mouse molars can most probably be applied to human tissue.

The purpose of this literature review is to give an account of those studies which have a direct bearing on the organization of the periodontal ligament and the ultrastructure of its components.

### 3.1 CELLS OF THE PERIODONTAL LIGAMENT

#### a. FIBROBLASTS

Bloom and Fawcett (1975) reported that fibroblasts are responsible for the production and long term maintenance of

extracellular connective tissue components. Deporter and Ten Cate (1980), determined that fibroblasts are not only involved in the turnover of extracellular proteins within the body of the ligament, but also actively remodel collagen at the alveolar bone-ligament and the cementum-ligament interfaces.

Within the ligament, fibroblasts exist in close apposition to ground substance, collagen and oxytalan fibres. Beertsen, Everts and van den Hooff (1974a) reported that when viewed with a TEM, oxytalan fibres appeared as a complex meshwork around periodontal fibroblasts in the tooth related part of rat incisor ligament.

Berkovitz and Shore (1982), stated that fibroblasts are orientated radially to the tooth surface in transverse section, whereas in sagittal section, fibroblast orientation varies within the ligament. Near the alveolar crest these cells are situated perpendicularly to the tooth surface, whereas they are arranged more obliquely to the tooth surface in the middle portion of the ligament. In the apical and interradicular areas, as well as in the loose connective tissue near nerves and blood vessels, fibroblasts are more randomly orientated to the tooth surface.

Despite difficulty in ascertaining cell shape using a TEM, many authors, including Fullmer (1967), Brunette, Melcher and Moe (1976) and Beertsen, Everts and Brekelmans (1979), have allocated specific cell shapes to periodontal fibroblasts. However, these cells are probably pleomorphic in form, their outline on histological section being dependent upon:

(i) the degree of cellular differentiation (Melcher, 1976);

(ii) the surrounding tissue substrate (Ross, 1968; Bloom and Fawcett, 1975);

(iii) the plane of section (Beertsen and Everts, 1977); and,

(iv) the effects of tissue processing (Weibel, 1979).

A scanning electron miscroscopic examination of rat molar periodontal ligament has been conducted by Roberts and Chamberlain (1978). These investigators concluded that, although variations exist between fibroblasts, these cells can be categorized into four morphologically distinct types, namely:

(i) oblong cells, 16 to 22 microns in length, orientated parallel to the principal collagen fibres, with roughened cell surfaces and no cell processes;

(ii) stellate cells, 8 to 13 microns in diameter, with multiple cell processes which occur in lacunar spaces between the principal fibres of ligament;

(iii) nodular, spheroid cells with a diameter of 7 to 12 microns being located near blood vessels; and,

(iv) elongate, stellate-shaped cells up to 60 microns in length, located adjacent to the principal fibres within the ligament and characterized by numerous pseudopodia-like cellular processes.

Although this study overcame some of the problems associated with TEM, Roberts and Chamberlain did not differentiate between fibroblasts and other cell types, and the techniques used doubtless caused considerable tissue distortion.

The literature is replete with descriptions of periodontal fibroblast ultrastructure. However, only those articles differentiating periodontal fibroblasts from other cell types, detailing features peculiar to periodontal fibroblasts, or documenting morphological variations of these cells, have been included in this review.

Periodontal fibroblast nuclei have been studied most comprehensively, using TEM, by Beertsen, Everts and van den Hooff (1974a), who studied the continuously erupting rat incisor, and Cho and Garant (1984), who compared the molar ligament of 5 week old and 20 month old mice. Beertsen, Everts and van den Hooff (1974a) found that, although generally ellipsoidal in cross-section, nuclear morphology varied with fibroblast location and cellular activity. More recently, Cho and Garant (1984) stated that nuclei of young mice are predominantly ovoid, whereas those of older mice are less regular in outline. They also determined that 17.6% of fibroblasts within the periodontal ligaments of 20 month old mice are multinucleated, and that 83% of these cells are located in the central portion of the ligament. More than 50% of the multinucleated pool contained at least four nuclei, 14% contained eight or more nuclei and at least one cell contained 17 nuclei. Some nuclei were predominantly euchromatic whilst other nuclei, in the same cell, contained mostly heterochromatin. These researchers suggested that this signified a variation in nuclear activation. Multinucleated cells contained multiple centrioles and were thought to arise by fusion of fibroblasts. The possibility that these cells were osteoclasts, or foreign body giant cells, was discounted because of their location within the ligament and the

presence of intracellular collagen profiles. Such cells are not reported in younger animals.

The only study to quantify nuclear volume in periodontal fibroblasts was conducted by Beertsen and Everts in 1977. Using a TEM and stereological principles they determined that the nucleus occupies 25% of the cell volume in 5 month old mice.

Mitochondria within periodontal ligament fibroblasts have been described by Beertsen, Everts and van den Hooff (1974a), Melcher (1976) and Berkovitz and Shore (1982), as being distributed randomly throughout the cell and present in relatively large numbers. Their profile varies from elongate to round. This variation possibly being an artifact of the plane of section.

Beertsen, Everts and van den Hooff (1974a) stated that some ribosomes occur as rosettes, while others form strands of endoplasmic reticulum that are well developed, located in the central parts of the cytoplasm and arranged in a parallel array. Beertsen and Everts (1977), quantified the volumetric proportion of granular endoplasmic reticulum, reporting that it comprises between 7 and 10% of the fibroblast cell volume. Cho and Garant (1984) reported that in older animals the rough endoplasmic reticulum is arranged more randomly and rarely demonstrates the parallel array seen in younger animals.

Garant and Cho (1979a) found the Golgi complex of periodontal fibroblasts situated to one side of the nucleus and adjacent to the rough endoplasmic reticulum within the cell. More recently, Cho and Garant (1984) stated that, in 20 month old mice, these organelles

became fewer in number, less prominent, and more randomly arranged within the cell.

The presence of microfilaments, within periodontal fibroblasts, was reported by Beertsen, Everts and van den Hooff (1974a). They found that these filaments are arranged in small bundles and located in the peripheral parts of the cytoplasm. These researchers postulated that such filaments are involved in cell locomotion because of their association with the cell membrane covering pseudopodia, lamellapodia and similar cell extensions. The plasma membrane associated with these cell processes, termed the undulating membrane, was considered to be the main locomotory organelle of single migratory cells by Abercrombie, Heaysman and Pegrum (1971). Recent findings by Fawcett (1981) suggest that the undulating membrane is associated with pinocytosis and hence microfilaments may be involved in this cell function. Farsi and Aubin (1983) further indicated that microfilaments, in the porcine periodontal fibroblast, are responsible for generating the force required to align and compact collagen.

Beertsen, Everts and van den Hooff (1974a) found that microtubules are orientated parallel to the long axis of some fibroblasts. They considered that these microtubules maintain cell shape, regulate cell mobility and are implicated in intracytoplasmic transport. This is consistent with later findings by Farsi and Aubin (1983) who reported that a change in microtubule distribution reflects a change in cell shape. Berkovitz and Shore (1982), stated that microtubules within periodontal fibroblasts are also associated with centrioles and cilia, centrioles being small cylinders of microtubules up to 2 microns in diameter.

Ciliated periodontal fibroblasts have been documented by a number of researchers. Beertsen, Everts and Houtkooper (1975), found solitary cilia lying within cell invaginations of approximately 70% of mouse incisor fibroblasts. These organelles are up to 2.3 microns in length and have a 9 + 0 microtubule arrangement which loses its ordered structure near the cilium tip. Some fibroblasts contained more than one cilium. Cho and Garant (1984) found cilia in multinucleated cells and reported that basal bodies are derived from centrioles that exhibit a nine-triplet arrangement and are located near the Golgi complex. Cilia, with a nine-doublet configuration, occur both singularly and in groups.

Collagen containing vacuoles have been reported within periodontal fibroblasts. Because collagen formation occurs extracellularly (Bloom and Fawcett, 1975), these vacuoles are considered to be sites of intracellular collagen degradation by many researchers including Ten Cate and Deporter (1975), Garant and Cho (1979a) and Berkovitz, Shore and Sloan (1980). Melcher (1976) postulated that cells containing collagen inclusions are a separate cell entity and termed these cells fibroclasts. These cells have since been regarded as fibroblasts in the more recent literature.

Beertsen, Everts and van den Hooff (1974a) described two types of intracellular collagen-containing vacuoles. The first type, characterized by the presence of beaded dilations, has the space between collagen fibrils and the surrounding limiting membrane filled with an electron-dense material. The fibrils in these vacuoles often lose their typical 640Å banding. The second type do not show

dilations, and the space between the collagen fibres and the unit membrane is filled with an electron-translucent material.

In 1979, Shore and Berkovitz found these two types of collagen containing vacuoles occurring within periodontal fibroblasts and stated that collagen fibrils within these vacuoles are orientated parallel to the adjacent extracellular collagen fibres. In addition, a third type of intracellular collagen profile, that is electron-lucent and contains fibrils with a diameter greater than that encountered extracellularly, was reported. Ten Cate, Deporter and Freeman (1976) had earlier suggested that different types of intracellular collagen vacuoles represent a sequence in collagen digestion. More recently, Shore and Berkovitz (1979) found that collagen containing vesicles are more abundant in rodent molar periodontal fibroblasts than in the ligament of the continuously erupting incisor, where the turnover rate of collagen is slower.

Berkovitz and Shore (1982) reported that desmosomes are the most frequent type of contact between periodontal fibroblasts. However, no information exists as to the distribution of these junctions within the ligament. Beertsen, Everts and van den Hooff (1974a), Azuma, Enlow, Fredrickson and Gaston (1975) and Frank, Fellinger and Steuer (1976), have reported that other cell contacts between fibroblasts include tight junctions and gap junctions. However, these researchers did not elaborate on their findings.

Beertsen, Everts and Brekelmans (1979) and Garant and Cho (1979a), determined that rodent periodontal fibroblasts not only exhibit the morphology of actively synthesizing cells, described

previously, but also the organelle polarity associated with cell migration. The nuclear, or proximal, end of the cell contains less cytoplasm, fewer profiles of granular endoplasmic reticulum and at least one narrow pseudopodal extension of cytoplasm. These extensions, also referred to as lobopodia, extend up to 10 microns in length and are generally less than 0.3 microns in diameter. Ultrastructurally, pseudopodia contain numerous cytoplasmic filaments and some microtubules. The distal end of the cell contains granular endoplasmic cisternae, smooth walled vesicles, secretion granules, dense bodies and numerous collagen containing vacuoles. The distal extremities of these cells often exist as trailing extensions of cytoplasm. Cho and Garant (1984), found that mononuclear periodontal fibroblasts in 20 month old mice were less highly polarized and more randomly orientated to the surrounding collagen fibres than fibroblasts in 5 week old mice.

Beertsen, Everts and van den Hooff (1974a), as well as Shore and Berkovitz (1979), had previously postulated that this cell polarity and the migratory potential of fibroblasts along substrata within the ligament, may be linked to tooth eruption. However, this view has not been substantiated.

The volume of fibroblasts within the periodontal ligament has been estimated by Berkovitz and Shore (1982) to be 50% of the extravascular tissue volume. Although these cells are the principal cells of the periodontal ligament, this figure is probably an overestimation as these authors assumed that all cells present within the ligament were fibroblasts.

The turnover of fibroblasts has been investigated by introducing tritiated thymidine into the experimental animal with osmotic minipumps. Garant and Cho (1979b) and Gould, Brunette and Dorey (1982), reported the turnover of periodontal cells is slow, with 42 days required to label 50% of the cells in the molar periodontal ligament. Gould, Melcher and Brunette (1980) and McCulloch and Melcher (1983) have postulated that fibroblasts arise from precursor cells located perivascularly. However, earlier findings by Garant and Cho (1979a) suggest that periodontal cells do not migrate from any stem cell area within the ligament. Melcher (1976) stated, that although fibroblasts exist in various stages of differentiation within the ligament, these cells ordinarily do not give rise to other cell types once they have become active.

Fibroblasts with a crenated nucleus and prominent microfilaments have been classified as myofibroblasts. These cells, once considered to be intermediate between fibroblasts and smooth muscle cells, are regarded in the current literature to be fibroblasts (Ohtani and Sasano, 1983). Although reported as occurring in the periodontal ligament by Azuma in 1975, the presence of these cells in periodontal tissue has recently been disputed by Berkovitz and Shore (1982). For these reasons, myofibroblasts have been included as fibroblasts in this study.

Rhodin (1968) classified some fibroblasts, located paravascularly near the venous circulation, as veil cells. Rather than simple cytoplasmic processes, these cells have sheets of flatly extending cytoplasm, 0.2 microns in diameter. Veil cells are otherwise

ultrastructurally equivalent to fibroblasts and have not been differentiated from fibroblasts in the present study.

Other fibroblasts, termed a sheath of Henle, have been found forming a delicate investment around nerve fibres in the periodontal ligament of cats by Bonnaud, Proust and Vignon (1978). These cells were considered to be part of subterminal nervous structures rather than receptors.

b. OSTEOBLASTS

Berkovitz and Shore (1982), stated that periodontal osteoblasts are located adjacent to the alveolar bone during active osteogenesis but are replaced by cells with little cytoplasm during periods of quiescence.

Ultrastructurally, these cells are similar to fibroblasts. However, the following features have been documented by Bloom and Fawcett (1975) and Berkovitz and Shore (1982) as indicative of osteoblasts:

(i) columnar or cuboidal cell shape;

(ii) a nucleus polarized away from the bone surface;

(iii) well developed Golgi apparatus, with associated vacuoles, situated between the cell nucleus and the alveolar bone;

(iv) prominent microfilaments near the bone surface; and,

(v) small lipid droplets and membrane bound dense bodies within the cytoplasm.

c. CEMENTOBLASTS

Berkovitz and Shore (1982), reported that active cementoblasts form a recognizable cell layer adjacent to cementum and the fine structure of these cells varies with their location in the ligament. Cementoblasts associated with the formation of cellular cementum matrix have cytoplasmic processes extending towards the tooth root, whereas those depositing mineralizing acellular cementum do not. These investigators concluded that apart from their proximity to the tooth root, cementoblasts are difficult to distinguish from periodontal fibroblasts.

Gurling (1982) stated that extensive rough endoplasmic reticulum, with associated vesicles containing an amorphous material and numerous mitochondria, are features indicative of cementoblasts.

d. OSTEOCLASTS

Osteoclasts are giant cells, up to 20 microns in diameter, that contain as many as 100 nuclei and are closely associated with areas of bone resorption. Berkovitz and Shore (1982), reported, that periodontal osteoclasts have a less conspicuous granular endoplasmic reticulum than periodontal osteoblasts and that microfilament bundles and microtubules are also sparse.

Bloom and Fawcett (1975) had earlier described the following structural features as characteristic of active osteoclasts:

(i) a cell polarity with the nuclei situated away from the bone surface;

(ii) a radially striated cell membrane adjacent to the bone, termed a ruffled border;

(iii) mitochondria located near the ruffled border but not adjacent to it;

(iv) numerous Golgi complexes located between the nuclei;

(v) small, dense, spherical membrane bound vesicles, 0.2 to 0.5 microns in diameter; and,

(vi) frequent lysosomes 0.5 to 3 microns in diameter.

Although these cells probably arise from the fusion of mononuclear cells, their precise origin still remains controversial. Some researchers including Tonna (1960), Rasmussen and Bordier (1974), and Wong and Cohn (1974) consider that osteoblasts and osteoclasts arise from the same cell population, although these workers do not agree as to whether osteoblasts arise from osteoclasts or vice-versa. Bloom and Fawcett (1975), in their text on general histology, suggested that a reversible modulation between osteoclasts, osteoblasts, osteocytes and osteoprogenitor cells occurs and hence any transformation between cell types merely represents a change in cell function. Conversely, Jee and Nolan (1963), and Gothlin and Ericsson (1973), produced evidence indicating that osteoblasts and osteoclasts are derived from different stem-cell populations. These researchers determined that osteoclasts originate from the fusion of haemopoietic cells, most probably monocytes or macrophages, whereas osteoblasts are of mesenchymal origin.

Roberts (1975), investigated the origin of periodontal osteoclasts and concluded that the increased cellularity, in response to bone resorption was partially accounted for by local cell proliferation and that an influx of migrating cells occurs.

e. PROGENITOR CELLS

Melcher (1976) described undifferentiated mesenchymal cells within the ligament as having a small nucleus and very little cytoplasm. The location of these cells has been investigated by McCulloch and Melcher (1983), who conducted an autoradiographic and histological study of the mouse molar periodontal ligament. These researchers determined that 40% of the proliferative cells are located within 10 microns of periodontal blood vessels. This is consistent with earlier findings by Gould, Melcher and Brunette (1977), and Freeman (1980) who further reported that some progenitor cells are located near the root surface.

Osteoprogenitor cells were described by Bloom and Fawcett (1975), as having an oval or elongate nucleus, little cytoplasm, few organelles and as being situated at or near the free surface of bone. Gould, Melcher and Brunette (1977), determined that the population of periodontal osteoprogenitor cells is perivascular in location.

Weiss, Stahl and Tonna (1968) labelled periodontal cells with tritiated thymidine and noted that the proliferative activity of cells is greatest within the body of the ligament, less adjacent to alveolar bone and least near cementum.

f. EPITHELIAL CELLS

Epithelial cells occur in the periodontal ligament as epithelial cell rests of Malassez. Gurling (1982), who examined mouse molar cementogenesis reported that epithelial cells arise from Hertwig's epithelial root sheath and, more coronally, from the reduced enamel epithelium.

The fate of epithelial cells in the rodent molar periodontal ligament is unclear. Shibata and Stern (1967), Lester (1969), Jande and Belanger (1970), and Freeman and Ten Cate (1971), have stated that epithelial cells become encapsulated by and degenerate within forming cementum. Conversely, Diab and Stallard (1965), who examined rat molar periodontal ligament, determined that at least some epithelial cells degenerate within the parenchyma of this tissue.

Melcher (1976) reported, that in the mouse molar periodontal ligament, the majority of the epithelial cells are incorporated into forming cementum, and consequently, few epithelial rests of Malassez are present. Any epithelial rests within the ligament are located close to, but not in contact with, the cementum and occur as strands, tubules and networks of cells parallel to the root surface. Spouge (1980), in his review of the literature, stated that epithelial cells do, on rare occasions, come into contact with cementum and in these regions epithelial cell aggregates are enlarged. However, he did not report in which animals this occurs.

Wentz, Weinmann and Schour (1950), who recorded the distribution of epithelial remnants in the rat molar periodontal ligament, reported that nearly 50% of the periodontal epithelial cells are located in the cervical region of the root. By contrast, Scott and Symons (1982), in their text on oral histology, have suggested that epithelial cells are most numerous around the apices of teeth. This disparity in findings possibly arises from a species or age difference of the animals examined.

Bevelander and Nakahara (1968), reported that human epithelial rest cells are irregular in outline, occur in close proximity to one another and have short villus-like projections that interdigitate with similar structures of adjacent cells. Lester (1969), stated that initially epithelial cells are surrounded by a basement membrane, have a clearly demarcated Golgi complex, with few associated membrane bound dense granules, and contain numerous scattered mitochondria. Tonofilament bundles and related desmosomal junctions, sparse endoplasmic reticulum, and ribosomes arranged as rosettes were also considered characteristic of periodontal epithelial cells. It was noted that as these cells matured, the cytoplasmic volume decreased, tonofilament bundles became more prominent and rough endoplasmic reticulum assumed the form of small dilated sacs. Nuclear pores became more marked and membranous vesicles near the Golgi apparatus dilated. Valderhaug and Nylen (1966), considered that the presence of lipid within human epithelial rests was an indication of cell degeneration.

The functions of the periodontal epithelial rests have not yet been resolved. Although originally thought by Robinsohn (1926, cited by Spouge, 1980), to have an endocrine function, recent studies by Ten Cate (1965) and Valderhaug and Nylen (1969) discount this. Löe and Waerhaug (1961), indicated that epithelial cells may be involved in the maintenance of the periodontal ligament and that these cells may protect the tooth root against resorption.

Gurling (1982) hypothesized, that if acellular cementum was damaged and epithelial cell rests contacted dentine, then it was theoretically possible for the epithelial cells to stimulate the formation of cementoblasts from periodontal fibroblasts.

g. VASCULAR AND PERIVASCULAR CELLS

Blood vessel walls are comprised of an endothelial cell lining with or without surrounding perivascular cells.

Avery, Corpron, Lee and Morawa (1975) examined the ultrastructure of endothelial cells in the molar ligament of 25 to 50 day old mice. They found that these cells contain many microvesicles, scattered ribosomes and small oval mitochondria. The endothelial lining of capillaries lack fenestrae, but have finger-like projections that extend into the vessel lumen. Tortuous intercellular junctions are characteristic of this endothelium.

Rhodin (1967, 1968), in his comprehensive articles on vascular wall morphology, described four types of specialized cells that are located perivascularly. These are:

(i) veil cells, described previously (page 14);

(ii) pericytes;

(iii) primitive smooth muscle cells; and,

(iv) smooth muscle cells.

Pericytes, primitive smooth muscle cells and smooth muscle cells were considered to be a progression of cell types, with primitive smooth muscle cells being intermediate to the other cell types.

Rhodin (1968) stated that, although pericytes resemble fibroblasts, pericytes have the following characteristics:

(i) are perivascular in location;

(ii) are at least partially covered by a basement membrane;

(iii) exhibit few intercellular contacts; and,

(iv) have pinocytotic vesicles located preferentially on the side of the cell nearest the vessel lumen.

Pericytes are most commonly seen in the vessel walls of venous capillaries, postcapillary venules and collecting venules up to 50 microns in diameter. Rhodin considered that the function of these cells includes:

(i) mechanical support to prevent collapse of the blood vessel;

(ii) protection against an excessive loss of blood elements;

(iii) the detection of damage to the endothelial cell lining;

(iv) the differentiation into phagocytic, smooth muscle and other perivascular cells; and,

(v) a contractile potential regulating regional blood flow.

He documented that primitive smooth muscle cells are similar to pericytes but completely surrounded by a basement membrane, frequently contact other primitive smooth muscle cells and contain an increased amount of intracellular filamentous material related to dense fusiform bodies.

Smooth muscle cells, associated with both the arterial and venous circulations, are spindle shaped, approximately 5 microns in width and up to 40 microns in length. The abundance of myofilaments and fusiform dense bodies in these cells obscure other intracellular organelles. These cells are surrounded by a basement membrane and occur either singularly, or as groups of cells arranged in layers within the blood vessel wall.

h. NEURAL AND PERINEURAL CELLS

Both myelinated and unmyelinated nerves occur within the periodontal ligament (Bernick, 1952; Simpson, 1966; Kubota and Osanai, 1977). However, some variation in neural ultrastructure occurs between species (van Steenberghe, 1979). Whereas, nonmyelinated nerves are embedded within Schwann cell cytoplasm and vary in diameter between 0.5 and 1 micron, myelinated nerves have a Schwann cell membrane coiled around the nerve axon and are between 1 and 16 microns in diameter (Ganong, 1975).

Corpron, Avery, Morawa and Lee (1974) used a TEM to examine nerve fibres in the mouse molar periodontal ligament. They found that small non-myelinated fibres exhibit either a Schwann cell coating or are covered by a thin cellular investment rich in microvesicles. Other fibres are separated from the surrounding collagen only by a thin basement lamina. The terminal ends of unmyelinated nerve axons are bulbous and contain an accumulation of mitochondria, microvesicles and dark granules.

Berkovitz and Shore (1978), reported that in some unmyelinated nerve fibres within the rat molar periodontal ligament, mitochondria almost obscure other cytoplasmic organelles. Nerve axons vary between 0.5 and 4 microns in diameter, with larger fibres usually situated close to the main neurovascular bundles. Smaller fibres are present in areas of dense collagen to within 25 microns of the cementum surface. Although these fibres branch, they course singularly within the ligament. Berkovitz and Shore (1978), did not ascertain if mitochondria rich unmyelinated nerves arose from larger unmyelinated nerves or represented the distal ends of myelinated fibres. However,

Griffin and Harris (1968) claimed, that human periodontal unmyelinated nerve fibres arise from myelinated ones which, having lost their myelin sheaths, divide into smaller branches surrounded by Schwann cells.

Fillenz (1971), reported that whereas sensory nerves contain only a single type of neurotransmitter granule, sympathetic nerve fibres contain two types of catecholamine storage particles and this feature can be used to distinguish between fibre types. Peters, Palay and Webster (1974), stated that although some sympathetic and parasympathetic nerve fibres are individually enclosed by Schwann cell cytoplasm, it is common for groups of autonomic axons to be enclosed in the same Schwann cell trough. As such axons approach their terminations, they gradually lose their cell investment, to be covered only by a basement membrane. Although species variation occurs, this arrangement was not reported for sensory nerves.

Griffin and Harris (1968), described the ultrastructural features of human periodontal Schwann cells as:

(i) marginal condensations of chromatin and several nucleoli within cell nuclei;

(ii) few free ribosomes and dilated profiles of granular endoplasmic reticulum;

(iii) numerous, small mitochondria;

(iv) a few electron-dense membrane-bound lysosomes;

(v) numerous, intracytoplasmic microfilaments and microtubules;

(vi) a basement membrane up to 50 nm thick; and,

(vii) occasional cilia.

Beertsen, Everts and van den Hooff (1974b), Berkovitz and Shore (1978) and Everts, Beertsen and van den Hooff (1977), reported another cell type, termed K-cells because of their kidney shaped nucleus, which occur adjacent to mitochondria rich unmyelinated nerves in rodent incisor periodontal ligament. As yet, these cells have not been illustrated in the mouse molar ligament.

Everts, Beertsen and van den Hooff (1977), stated that K-cells in mouse incisor periodontal ligament have a rounded cell body with few cytoplasmic extensions, are surrounded by a basement membrane and have an eccentrically placed nucleus. Ultrastructurally, there is much granular endoplasmic reticulum throughout the cell. A prominent Golgi apparatus is located near the cell nucleus and microtubules radiate from the centriolar region into cytoplasmic processes. Microfilaments 5 to 7 nm in diameter are present in the peripheral cytoplasm, particularly within cell processes where a filamentous material, with periodicity of 160 nm, is located. Furthermore, many vesicles, 60 nm in diameter are present beneath the cytoplasmic membrane, predominantly on the neural and connective tissue interfaces of the cell. Zonulae occludentes occur between adjacent K-cells.

Beertsen, Everts and van den Hooff (1974b) and Berkovitz and Shore (1978), noted that K-cells in the rat incisor periodontal ligament contained fibrillar cross-banded structures, called leptomeric organelles. These organelles have a periodicity varying between 100 and 170 nm and this variation was interpreted as indicative of the cell being stretched. Because of this, it was postulated that K-cells have a receptor function. However, these cells have numerous mitochondria, much rough endoplasmic reticulum, a prominent Golgi complex and many

peripheral membrane covered vesicles, characteristics of actively synthesizing cells.

i. MACROPHAGES

Berkovitz and Shore (1982) stated that the fine structural appearance of periodontal macrophages depends on their state of activity and that resting macrophages are difficult to distinguish from periodontal fibroblasts. The ultrastructural features present in macrophages that distinguish them from active fibroblasts are:

(i) characteristic lysosomes of varying size and electron density;

(ii) a U-shaped nucleus with invaginations of electron dense chromatin condensations;

(iii) an extensive Golgi apparatus;

(iv) a few ribosomes arranged either singularly, as rosettes, or as short strands of granular endoplasmic reticulum; and,

(v) peripheral cytoplasmic processes, microvilli and finger-like projections of varying size and shape.

## 3.2 FIBRES OF THE PERIODONTAL LIGAMENT

### a. COLLAGEN

Periodontal collagen is generally accepted to be arranged in definite fibre bundles which are embedded, by means of Sharpey's fibres into alveolar bone on one side of the ligament and cementum on the other. Melcher (1976), Beertsen and Everts (1977) and Freeman (1980) documented that the majority of periodontal collagen is organized as fibre bundles arranged radially to the tooth in five principal fibre groups, namely:

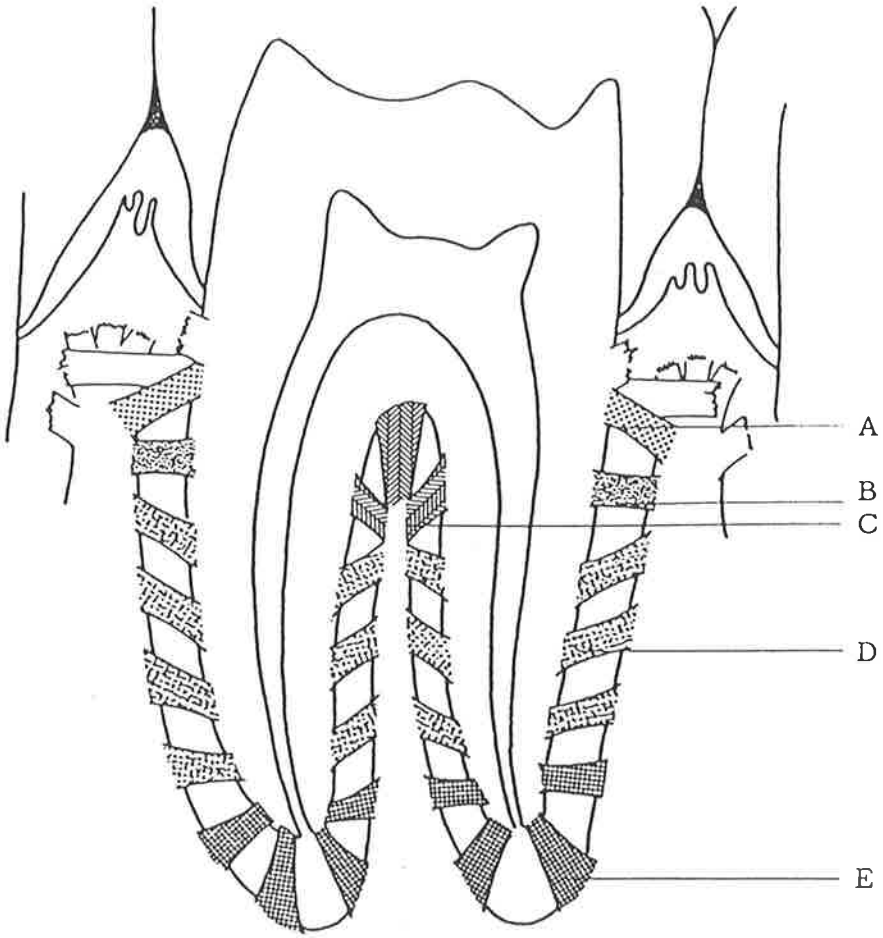


Figure 1. The arrangement of periodontal collagen into groups of principal fibre bundles (Freeman, 1980).

A - Crestal fibres
B - Horizontal fibres
C - Interradicular fibres
D - Oblique fibres
E - Apical fibres

(i) oblique fibres;

(ii) alveolar crestal fibres;

(iii) horizontal fibres;

(iv) interradicular fibres; and,

(v) apical fibres.

However, Sloan (1982) reported finer collagen fibres, arranged in an apparently unorientated fashion, occurring as a meshwork around periapical fibre bundles, blood vessels and nerves. Other researchers, including Shackleford (1971, 1973), have disputed this arrangement of fibres, but their results cannot be correlated with any other studies and are most probably an artifact of preparation (Ten Cate, Deporter and Freeman, 1976; Sloan, 1982).

Reports on the turnover rate of rat and mouse molar periodontal collagen indicate that a half-life of between 2 and 6.5 days (Rippin, 1976; Berkovitz, Weaver, Shore and Moxham, 1981), a rate which Ten Cate and Deporter (1975) consider is probably faster than in any other region of the body. In contrast, Sloan (1982) reported the half-life of periodontal collagen may be as long as 23 days. Rippin (1976) and Ten Cate, Deporter and Freeman (1976) have indicated that the turnover of collagen is not confined to any one region within the ligament. However, Wiebkin (1983) considers that type III collagen, which constitutes 20% of bovine periodontal ligament (Butler, Birkedal-Hansen, Beegle, Taylor and Chung, 1975), is remodelled at the greatest rate.

In the rat molar ligament, collagen fibrils average 43 nm in diameter, although much variation occurs between adjacent fibre bundles

and within the same collagen bundle (Berkovitz, Weaver, Shore and Moxham, 1981). These investigators also reported that only 35% of these fibre bundles are in fact occupied by fibrils and that ground substance constitutes the majority of the collagen fibre volume.

b. ELASTIC FIBRES

Fullmer (1960), Fullmer, Narkates and Sheetz (1974) and Melcher (1976) reported that elastic fibres are abundant in the periodontal ligaments of some animals including rabbits, deer, dogs, sheep and swine. In other animals, including mice, oxytalan fibres have been found instead of elastic fibres, with any elastic fibres present being confined to blood vessel walls.

c. OXYTALAN FIBRES

Carmichael (1968) described oxytalan fibres in the mouse molar periodontal ligament as traversing from cementum to periodontal vessels and as usually being perpendicular to the occlusal plane. He reported that some fibres ran at right angles to collagen fibres and a continuity, or extremely close contiguity, occurs between oxytalan fibres and cell processes.

Edwards (1968) noted an increase in the size and number of oxytalan fibres in beagle dogs following the orthodontic rotation of incisors and stated that oxytalan fibres were most numerous in regions of increased stress.

Sims (1973), reported an oxytalan fibre meshwork in the mouse mandible which extended from mesial of the first molar to the distal of the third molar, terminating at its coronal limit adjacent to well

defined condensations of muscle and elastic fibres. Within the ligament, oxytalan fibres are orientated in five principal patterns around the root (Figure 2).

Fibre tracts extend apically to enter blood vessel walls and curve beneath the root apex to form a fine irregular mesh. These oxytalan tracts receive numerous additional fibres from the adjacent cementum. It was hypothesized that oxytalan fibres constitute part of a receptor mechanism that regulates vascular flow according to functional tooth movements.

Following histological investigation of mouse and human tissue, Sims (1975), reported oxytalan fibres form a dense three dimensional plexus between the dentino-cemental junction of the tooth and periodontal vessels. Whilst some fibres are randomly associated with individual vessels, other fibres form a network linking arteries, veins and lymph vessels within the ligament. In 1977, further histological examination of the mouse molar periodontal ligament revealed that the oxytalan fibre meshwork does not extend into the alveolar bone. More recently, Sims (1983) reported that the juxtaposition of periodontal nerve fibres and oxytalan fibres provides additional evidence that oxytalan fibres form part of a proprioceptor system.

Edmunds, Simmons, Cox and Avery (1979), examined the distribution of oxytalan fibres within the periodontal ligament of guinea pigs using histological and electron microscopic techniques. They found an interweaving of oxytalan filaments with collagen fibres and suggested that oxytalan fibres may reinforce tissue structure.

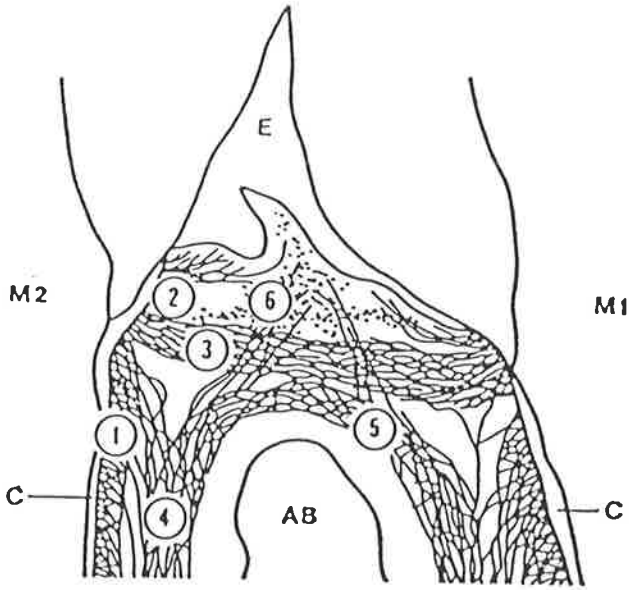


Figure 2. Schematic representation of the oxytalan fibre meshwork (Sims, 1973).

C - Cementum
E - Epithelium
B - Alveolar Bone
1 - First mandibular molar
2 - Second mandibular molar

1. a delicate intercommunicating plexus alongside cementum;
2. fibres curving upwards towards the interdental papilla;
3. horizontal fibres dividing and anastomosing across the transeptal region to communicate with similar fibres from the approximating tooth;
4. fibres which, after emerging from the cementum, increased in diameter as they changed direction to an apico-occlusal direction and extended apically;
5. a prominent fibre group that curved over the interseptal alveolar crest and established continuity between group 4 of adjacent teeth; and,
6. fibres passing interproximally from the buccal to lingual interproximal papillae.

Although much of the research into oxytalan fibres has been centred on the periodontal ligament, there is increasing evidence that these fibres are located elsewhere in the body. Fullmer, Sheetz and Narkates (1974), stated that oxytalan fibres have been found in human tympanic membrane, giant cell tumours of tendon sheaths, sclerosing haemangiomas of the skin and dermal fibromas, but that these findings have not been substantiated with a TEM. More recently Booij and Markens (1983) reported oxytalan fibres in subcutaneous connective tissue, tumour capsules, cranial sutures, gingival tissue, pulpal tissue and the walls of blood vessels. Using histological techniques, they demonstrated oxytalan fibres in the articular disk of the rat temporomandibular joint and an increase in fibre number under certain experimental conditions. These researchers postulated that oxytalan fibres increased cohesion of collagen, thus providing resistance to distortion.

Following a histochemical study of human oral mucosa, Sastry (1983) reported that oxytalan fibres are present within oral mucosa and increase in number during the early stages of oral submucous fibrosis. Calvo and Boya (1983), who examined rat pineal gland, found that oxytalan fibres in this tissue lack transverse striations and the number of fibres increases with age. Goldfischer, Coltoff-Schiller, Schwartz and Blumenfeld (1983), have suggested that microfibrils present in the aorta are analogous to periodontal oxytalan fibres. None of these researchers suggested a function for these fibres.

Carmichael and Fullmer (1966) and Sloan (1982), determined that oxytalan fibres are an assemblage of fibrils 5 to 15 nm in diameter orientated parallel to the oxytalan fibre axis and interspersed with a

variable amount of an amorphous interfibrillar material. The diameter of oxytalan fibres in the developing human periodontium has been reported by Griffin and Harris (1967) to be between 125 and 350 nm, a figure which is slightly less than the diameter of mature fibres in the mouse molar periodontal ligament recorded by Sims (1973, 1981). In the latter study, Sims used a TEM and found that their cross-sectional size and shape is variable. The fibres are rarely circular in section and frequently have a flattened, irregular appearance particularly in the occlusal portion of the ligament. The major axis of oxytalan fibres varies between 200 and 6900 nm and the minor axis between 100 and 1000 nm. Although variation of the average value of the major axis of these fibres with depth is statistically significant, the functional significance of these findings has not been ascertained.

## 3.3 GROUND SUBSTANCE

Although not readily apparent on TEM sections (Freeman, 1980), ground substance is present throughout the periodontal ligament (Melcher, 1976) and comprises 65% of the collagen fibre bundle volume in the rat periodontal ligament (Berkovitz, Weaver, Shore and Moxham, 1981). Melcher (1976) and Freeman (1980) determined that 70% of ground substance is water and is bound mainly to proteoglycans and glycoproteins.

The functions ascribed to ground substance by Fawcett (1981) and Connor, Aubin and Melcher (1983) include those of the glycoprotein fibronectin, namely:

(i) maintenance of soft connective tissue;

(ii) attachment to mineralized surfaces in the periodontium;

(iii) cell to cell adhesion; and,

(iv) cell to substrate adhesion.

Many researchers including Parfitt (1960) and Picton (1963) have implicated periodontal ground substance in supporting the tooth against intrusive and lateral loading forces. Although it is not clear how this occurs, it has been postulated by many researchers, including Bien and Ayers (1965), Bien (1966), Wills, Picton and Davies (1976) and Walker (1980), that the vascular system is probably involved. Alternatively, Kardos and Simpson (1979, 1980) suggested that periodontal collagen and associated ground substance constitute a collagenous thixotropic matrix which is involved in tooth support, tooth eruption, functional adaptation to forces applied to teeth and changes that occur during orthodontic tooth movement. More recently, Ferrier and Dillon (1983) reported that the amount of water bound to glycoproteins and proteoglycans is altered when teeth are loaded and determined that fluid mechanisms support the tooth against forces of less than 0.1 kg. They considered that forces greater than this are resisted by the collagen fibre network. These findings are in agreement with those of Wills, Picton and Davies (1976).

Orlowski (1978), examined rat incisor periodontal ligament and found that non-collagenous proteins have a half-life of 6.5 days. Because the turnover of periodontal cells is slow (Gould, Brunette and Dorey, 1982), Orlowski's results indicate a rapid turnover of ground substance.

## 3.4 BLOOD VESSEL ULTRASTRUCTURE

Rhodin (1967, 1968) documented a comprehensive classification of arterial vessels (Table 1), and venous vessels (Table 2) present in rabbit thigh fascia on the following criteria:

(i) the lumen calibre;

(ii) the endothelial cell lining;

(iii) the patterns of vessel branching and anastomosis; and,

(iv) the constituents of the blood vessel wall.

Capillary types have been classified by Bennet, Luft and Hampton (1959), Forsslund (1959), Herdson (1967) and Wolff (1977). Of these classifications, the system documented by Bennet, Luft and Hampton (1959), is the most frequently cited in the periodontal literature, and is based on the nature of the endothelial cell lining, the existence or absence of a continuous basement membrane and degree of perivascular cellular investment. The following notation is used:

Type A: a continuous basement membrane;

Type B: without a continuous basement membrane;

Type 1: capillaries without fenestrae or perforations;

Type 2: capillaries with intracellular fenestrations;

Type 3: capillaries with intracellular perforations;

Type $\alpha$: capillaries without a complete pericapillary cellular investment;

Type $\beta$: capillaries with a complete pericapillary cellular investment.

Consequently, using this system, blood vessels in the mouse molar periodontal ligament, described by Avery, Corpron, Lee and

| Blood Vessel Type | Lumen Calibre | Wall Thickness | Endothelial Cell Morphology | Peri-endothelial Cells | Other |
|---|---|---|---|---|---|
| Arteriole | 100-50$\mu$m | Greater than 6$\mu$m | Cell 0.15 to 2$\mu$m in width, few pinocytotic vesicles, upstream cell usually overlaps downstream cell. | 2-3 layers of smooth muscle cells, some eosinophils, mast cells and macrophages. | Well developed elastica interna, non-myelinated nerves extending to smooth muscle layer. |
| Terminal Arterioles | Less than 50$\mu$m | Less than 6$\mu$m | Generally as above but with many filaments parallel to the long axis of the blood vessel and with more pinocytotic vesicles. | One layer of smooth muscle cells. | Little elastic interna, nerves closer to vessel wall with more frequent contacts with the smooth muscle layer, some myoendothelial junctions. |
| Precapillary | 7-15$\mu$m | Less than 5$\mu$m | Cell protrudes towards vessel lumen, nucleus shorter, thicker and more lobulated than above, some cytoplasmic filaments, many pinocytotic vesicles. | One layer of smooth muscle cells. | An increased number of unmyelinated nerves associated with a decrease in lumen diameter, frequent neuromuscular and myo-endothelial junctions. |

TABLE 1: Ultrastructural features of arterial blood vessels. (Rhodin, 1967)

| Blood Vessel Type | Lumen Calibre | Wall Thickness | Endothelial Cell Morphology | Peri-endothelial Cells | Other |
|---|---|---|---|---|---|
| Venous Capillary | 4-7$\mu$m | 0.3-1.3$\mu$m | Some rough endoplasmic reticulum, free ribosomes, mitochondria, vesicles, granules and filaments. | Occasional veil cells and pericytes. Some macrophages, leukocytes, lymphocytes and plasma cells. | Endothelium may be fenestrated. |
| Post-capillary venule | 8-30$\mu$m | 1-5$\mu$m | Cell rarely less than 0.2$\mu$m thick and generally larger than that of venous capillary. Slight over-lapping of adjoining cells. | More pericytes and veil cells than above. Some primitive smooth muscle cells around larger vessels. | Endothelium generally lacks fenestrae. Leukocytes may adhere to endothelial wall. |
| Collecting Venule | 30-50$\mu$m | 1.7$\mu$m | As above. | Continuous layer of pericytes and veil cells around vessel. More primitive smooth muscle than above. Smooth muscle cells around larger vessels. | Single layer of veil cells and some collagenous fibrils surround blood vessels. |
| Muscular Venule | 50-100$\mu$m | 2.0$\mu$m | As above. | 1-2 layers of smooth muscle cells. | Veil cells form a complete layer around vessel wall. Myoendothelial junctions present. |
| Small collecting vein | 100-300$\mu$m | 2-3$\mu$m | As above but with specific endothelial granules. | 2 or more layers of smooth muscle cells. | Unmyelinated nerves situated 5-10 microns from muscular layer. |

TABLE 2: Ultrastructural Features of Venous Blood Vessels. (Rhodin, 1968)

Morawa (1975) as surrounded by a distinct basement lamina, incompletely surrounded by pericytes and without fenestrae, are classified as A-1-$\alpha$ capillaries.

The structure of blood vessels within human periodontal ligament was investigated by Bevelander and Nakahara (1968). They reported that periodontal vessels are thin walled, vary greatly in lumen calibre and are separated from surrounding fibroblasts and collagen fibres by a basement membrane.

Avery, Corpron, Lee and Morawa (1975), in a TEM examination of mouse molar ligament, found that some capillaries are surrounded by smooth muscle cells in 25 to 50 day old mice. Precapillary vessels, that exhibit a thicker endothelial lining and an incomplete muscular coating, are present throughout the ligament. The muscular layer is separated from the endothelium and the surrounding connective tissue by a distinct basement lamina which is breached at myoendothelial junctions.

A later abstract by Corpron, Avery, Morawa and Lee (1976), stated that vessels located adjacent to osteoblasts have fenestrae 30 to 50 nm in diameter, whereas capillaries located centrally within the mouse molar periodontal ligament do not. They also reported small arterioles located near alveolar bone and cementum which display an incomplete muscular coating. However, the criteria upon which vessel classification was undertaken, was not documented.

Roberts and Chamberlain (1978), utilized scanning electron microscopy to examine rat molar periodontal ligament and reported that sinusoids, capillaries and arterioles are present within this tissue.

Because Roberts and Chamberlain are the only researchers who have reported sinusoids within the periodontal ligament, this finding may be an artifact of tissue preparation.

Gilchrist (1978), following a TEM examination of buccal biopsies of human periodontal ligament, concluded that, because periodontal vessels are a venular microcirculatory system, earlier classifications of blood vessels by Bennet, Luft and Hampton (1959) and Rhodin (1967, 1968) are inappropriate for this tissue. He also reported that endothelial fenestrae are uncommon and, although some vessels are surrounded by an incomplete layer of pericytes, smooth muscle cells are not present within the ligament.

## 3.5 PERIODONTAL BLOOD VESSEL DISTRIBUTION

The vascular distribution within the mouse periodontal ligament is poorly documented. However, Carranza, Itoiz, Cabrini and Dotto (1966) have documented that the vessel architecture in mice and other more extensively studied laboratory animals, in particular the rat, are similar.

Castelli and Dempster (1965) perfused monkeys with India ink, Teichman's paste and radiopaque fluid and examined the periodontal vasculature using histological and microradiographic techniques. They found that whereas a layer of capillaries is located near cementum, venules form an irregular plexus closer to the alveolar bone. The venous plexus that drains towards the tooth apex, through the cribriform walls of the alveolus and via anastomoses with gingival vessels, is less regular in the apical and cervical thirds of the ligament than in the middle portion.

Carranza, Itoiz, Cabrini and Dotto (1966) used histochemical techniques and reported a similarity in the vessel distribution in the ligaments of rats, mice, hamsters, cats and dogs. Larger vessels, from the inferior dental artery, are most frequently located in the apical two-thirds of the ligament and traverse parallel to the long axis of the root, nearer to bone than cementum. From these vessels branch smaller ones that form an intertwining plexus around the root. Although some connections with pulpal vessels occur in the apical and bifurcation regions of the ligament, anastomoses with gingival blood vessels are rare. These findings are consistent with those by Boyer and Neptune (1962), Kindlová and Matena (1962) and Kindlová (1967) who investigated rat periodontal vasculature using light microscopic techniques, and Kindlová (1965) who conducted optical evaluation of monkey periodontal ligament.

Birn (1966) examined the number and size of perforations in the alveolar wall of human cadavers and, on the unvalidated assumption that each perforation contains a blood vessel, concluded that the vascular supply to the human periodontal ligament is greatest in the cervical third. This is contrary to the findings of Castelli and Dempster (1965), and Carranza, Itoiz, Cabrini and Dotto (1966) who found the apical region to be the most vascular.

Folke and Stallard (1967) introduced plastic microspheres, with a diameter of 15 ± 5 microns, into the periodontal circulation of squirrel monkeys to study the vascular pattern of these animals. Vessels were mainly apico-occlusal in orientation or formed glomeruli-like structures. Few plastic spheres were present within the

periodontal circulation and these researchers postulated that this occurred because:

(i) a regulatory mechanism that controls blood flow is present within the ligament;

(ii) of preferential vascular channels within the ligament; or,

(iii) of a progressive increase in the lumen diameter of periodontal vessels, as is seen in the venous return.

Garfunkel and Sciaky (1971) traced rat periodontal vessels, previously infused with India ink, using histological sections 100 to 280 microns thick. They reported that these vessels form a hammock-like network around the tooth root and are linked via supraperiosteal and bone marrow channels to the ligament surrounding adjacent teeth.

Possibly the most comprehensive analysis of the mouse molar periodontal vascular pattern has been conducted by Wong (1983), who examined methyl methacrylate castings of the microvascular bed using scanning electron microscopy. He reported occluso-apically orientated vessels that lie within regularly and widely spaced indentations in the socket wall. Although these vessels are the predominant type found, a circular ring of vessels, arranged in a glomeruli-like pattern, is also present near the alveolar margin. Polygonal anastomoses, from which short vascular branches arise to link with medullary vessels, are accompanied by an intertwining capillary network in the cervical region of the ligament. In the middle third, the vascular network is characterized by axially orientated venous vessels connected to the arterial circulation by capillary-like loops. A precapillary sphincter is associated with the anastomoses of these two vessel types.

In the apical third, larger venous vessels, approximately 26 microns in diameter, are organized in a hammock-like network around the tooth apex.

## 3.6 LYMPHATICS

Casley-Smith (1977), stated that without prior injection of tracers, lymphatic vessels are difficult to differentiate from blood vessels. However, lymphatic capillaries:

(i) usually are larger than blood capillaries;

(ii) have an irregular outline;

(iii) have abluminal projections, where collagenous filaments attach to endothelial cells;

(iv) exhibit frequent open junctions;

(v) contain fewer red blood cells and plasma proteins than blood vessels;

(vi) have a tenuous basement membrane which is absent in regions of high lymphatic activity;

(vii) usually are partially or totally collapsed;

(viii) have a thicker endothelial lining which appears thinner because of the increased lumen diameter; and,

(ix) have an endothelial cell lining that lacks fenestrae and normally stains less intensely than the endothelial cell lining of blood vessels.

Furthermore, the endothelial lining of lymphatic collecting vessels differs from similarly sized blood vessels because it contains ropheosomes with a diameter of approximately 200 nm and smooth vesicles, about 70 nm in diameter which occupy about 35% of the non-nuclear cytoplasmic volume. Bloom and Fawcett (1975) later stated

that whereas pericytes are frequently situated near blood capillaries, they are not found near lymphatic vessels.

The existence of lymphatic vessels in the periodontal ligament has been debated. Levy and Bernick (1968) examined 20 to 30 micron thick histological sections of marmoset periodontal ligament. These sections revealed that lymphatic vessels originate as blind endings within the body of the ligament. Lymphatic capillaries empty into collecting vessels which contain valves, are thin walled and surrounded by scattered fibroblasts and collagen fibres. Accompanying veins are free of valves and contain more red blood cells. Lymph vessels that arise near the alveolar crest pass, with adjacent blood vessels, over the bony crest and into the gingival and palatal mucosa. Vessels located in the middle of the ligament perforate through the alveolar bone whilst other vessels course within the ligament towards the tooth apex.

Ruben, Prieto-Hernandez, Gott, Kramer and Bloom (1971) demonstrated periodontal lymphatic channels in dogs by means of retrograde perfusion with carbon solution. They determined that numerous lymphatic vessels course apically on the alveolar side of the ligament and that some pass into the periosteum and the perivascular intraosseous channels. Quantitatively, the volume of lymphatics, is much less than that of the vascular system. Because of the proximity of cervical lymph nodes to the thoracic duct, it is possible that some vascular channels may have filled with carbon solution, giving misleading results. Vessels resembling lymphatic vessels have been reported in human tissue by Gilchrist (1978), however, lymphatic vessels have not been ultrastructurally confirmed in mice.

## 3.7 NEURAL DISTRIBUTION

Although it is generally accepted that periodontal innervation arises from the trigeminal nerve, the actual distribution of nerves within the ligament has not been resolved and may be species specific (van Steenberghe, 1979).

Van der Sprenkel (1936), was the first to examine the innervation of the mouse periodontium. Using histological techniques he found that the majority of periodontal nerve fibres arise from myelinated nerves that enter the periodontal ligament through the socket wall. Fibres arising from the base of the tooth socket ascend towards the gingiva and intermingle with nerves from the alveolus. Some fibrils were said to penetrate the layer of cementoblasts and terminate in dentinal tubuli. These findings are dissimilar to those by Lewinsky and Stewart (1936, 1937) who examined human and feline tissue, but are consistent with histological observations of monkey periodontal ligament by Bernick (1952).

In 1956, Bernick stated that in rat molar ligament, nerves arising from the base of the tooth socket do not communicate with nerves from the socket wall and terminate either as free nerve endings among cells and collagen fibres or pass into the surrounding gingival tissue.

Physiologically, Berkovitz and Shore (1978) and Hannam (1982) stated that parasympathetic nerves are not present within the periodontal ligament, but did not substantiate their opinions. They reported that sympathetic fibres are mostly unmyelinated, 0.2 to 1 micron in diameter and mingle to form a basket-like arrangement

around blood vessels. Griffin and Harris (1968), Bernick and Levy (1968) and Hannam (1982) considered that such nerves may regulate regional blood flow within the ligament. Furthermore, Berkovitz and Shore (1978) stated that any nerves morphologically dissimilar to sympathetic fibres, are sensory in function.

## 3.8 NERVE ENDINGS

Hannam (1982) reported that receptors within the periodontal ligament can be classified as free nerve endings and organized encapsulations. However, the exact nature of these nerve endings at the ultrastructural level is not clear and seems to be variable between species. Nerve endings within the ligament have been reviewed as follows.

### a. MOUSE

Van der Sprenkel (1936) described three kinds of terminations in the mouse periodontal ligament. These being:

(i) small end rings located adjacent to collagen fibrils near the alveolar wall;

(ii) nerve fibres that terminate as a reticulum around cell nuclei; and,

(iii) nerve endings terminating in dentinal tubuli.

Using a TEM, Corpron, Avery, Lee and Cox (1974) examined the ultrastructure of nerve endings in mouse molar periodontal ligament. They observed large encapsulations, 3 to 6 microns in diameter, present throughout the ligament and reported that these organized nerve terminations are surrounded by a single layered capsule, contain 2 to 4 nerves, and may have small unmyelinated nerves lying outside of the

capsular wall. Other bulbous nerve endings, with many mitochondria and surrounded by a cell coating rich in microvesicles, were also reported.

In mouse incisor ligament, K-cells, found in close apposition to nerve fibres by Everts, Beertsen and van den Hooff (1977), were considered to be part of a proprioceptive system.

More recently, Corpron, Avery, Lee and Morawa (1980) conducted a TEM examination of the molar periodontal ligament of 100 day old mice and found two types of receptors. Firstly, free nerve endings, considered typical of pain receptors, were located throughout the ligament. Secondly, unmyelinated nerve axons with a thin, discontinuous Schwann cell covering and numerous mitochondria, neurofilaments and microvesicles, were presumed to be a type of Golgi-Mazzoni mechanoreceptor. In this receptor type, Schwann cells often enclosed small pockets of fine collagen fibres distal to the nerve axon.

b. RAT

Beertsen, Everts and van den Hooff (1974b) and Berkovitz and Shore (1978), both studied the periodontal ligament of the continuously erupting rat incisor and reported K-cells in close apposition to nerve terminations. These cells, which are similar to K-cells in mice, are present only in the alveolar zone of the rat incisor ligament (Beertsen, Everts and van den Hooff, 1974b).

More recently, following a TEM examination of incisor ligament, Berkovitz, Shore and Moxham (1983) documented a lamellated corpuscle, with a diameter of 15 microns, situated near blood vessel endothelium.

This receptor, characterized by circularly arranged cell processes, was considered to be a rapidly acting mechanoreceptor.

c. CAT

Lewinsky and Stewart (1937), reported free nerve endings in the periodontal ligament near the tooth surface as well as spindle-like end organs arising from thick nerve fibres near the alveolar bone. This latter receptor type may represent Vater-Pacini corpuscles seen by Bonnaud, Proust and Vignon (1978), who used a TEM to examine cuspid periodontal ligament.

d. JAPANESE SHREW MOLE

Following light microscopic investigation, Kubota and Osanai (1977) concluded that the apical innervation in the Japanese shrew mole is far denser than that of the intermediate zone, and the ratio of these relative densities in the upper dentition is greater than that for the lower teeth. In the ligament of the lower first molar mesial root, the apical region has three times the innervation of the intermediate zone. The ligament is devoid of specialized nerve endings and free nerve endings form a basket-like plexus around the tooth apex with many free nerve endings located near cementum. The bifurcation region is poorly innervated.

e. MONKEY

Bernick (1952) reported that when viewed light microscopically, specialized nerve endings are not present within monkey molar periodontal ligament and that nerves terminate as free endings in the body of the ligament, among cementoblasts and within cementum. A similar distribution of free nerve endings was documented in marmoset

periodontal ligament by Bernick and Levy (1968) who also found the bifurcation region to be well innervated. Button-like enlargements at some free nerve endings and elongate club-like structures at the terminations of myelinated nerve fibres were present in the apical third of the ligament.

f. HUMAN

Nerve endings in human periodontal ligament were first described by Dependorf (1913, cited by Lewinsky and Stewart, 1936). Although Held and Baud (1955) stated that organized nerve endings are not present within the human periodontal ligament, this opinion is not substantiated by other studies which used light microscopy (Lewinsky and Stewart, 1936) or a TEM (Griffin and Harris, 1968, 1974a, 1974b; Harris and Griffin, 1974a, 1974b). However, Simpson (1966) considered that organized nerve terminations are uncommon.

Conversely, there are numerous free nerve endings within human periodontal ligament (Lewinsky and Stewart, 1936; Held and Baud, 1955 and Simpson, 1966). These occur either singularly, as a network of nerve endings within collagen bundles, or close to the nuclear membranes of fibroblasts. Griffin and Harris (1968) determined, using a TEM, that these endings are characterized by microvesicles and small mitochondria.

Lewinsky and Stewart (1936) described knob-like terminations within the ligament, following histological examination of extracted tissue with alveolar attachment. Griffin and Harris (1974a, 1974b), and Harris and Griffin (1974a, 1974b) confirmed these findings with a TEM and reported three types of organized nerve terminations.

Firstly, structures considered to be simple mechanoreceptors, are derived from myelinated nerve fibres 2 to 3 microns in diameter and course to the distal portion of the capsule where they divide into numerous unmyelinated nerve fibres characterized by synaptic-like vesicles, neurotubules and small mitochondria. Secondly, compound receptors, thought to detect stretch, arise from three or more myelinated nerves and numerous unmyelinated ones. These nerve complexes are characterized by capsular cells that enclose collagen fibres orientated parallel to the long axes of the unmyelinated nerve fibres. Thirdly, complex mechanoreceptors, considered to be baroreceptors, occur as encapsulations of myelinated nerves and adjacent metarterioles. These receptors, with associated free nerve endings located peripherally to the neural complex, were thought not to have an autonomic function because of the presence of myelinated fibres. However, sympathetic fibres may be myelinated (Bloom and Fawcett, 1975) and because this study used a small sample size, the results may be open to misinterpretation.

## 3.9 VOLUMETRIC PROPORTIONS

Literature quantifying the volumetric proportions of the various constituents of the periodontal ligament is limited.

Götze (1965) determined, from histological sections of human cadaver material, that the vascular volume comprises 1 to 2% of the periodontal ligament and decreases with age. This conclusion is consistent with findings by Parfitt (1967), who used clinical measurements and animal experiments to investigate tooth support. Wills, Picton and Davies (1976), using monkeys and a mathematical

model, determined the vascular proportion is 0.5 to 1% of the ligament volume. However, numerous assumptions made in deriving their results, were not substantiated experimentally.

In 1976, Gǫ̈tze determined that the volume proportion of periodontal blood vessels surrounding human anterior and premolar teeth varies between 1 and 4%. He found the proportion of vessels increases from the cervical region to the tooth apex and that the lingual and labial aspects are more vascular than the corresponding mesial and distal ones (Figure 3).

In contrast to these findings, Sims (1980) suggested that the vascular proportion in some regions of the periodontal ligament is much greater, being 17% in the mouse molar ligament, 11% in human mandibular premolar ligament and reaching 20% along the buccal aspect of maxillary premolars.

McCulloch and Melcher (1983) made an incidental finding that the volume proportion of periodontal ligament mesial to the first mandibular molar is 7.25 ± 0.75%. This finding is consistent with earlier work by Gould, Melcher and Brunette (1977) who reported that the vascular proportion of this tissue is 7.7 ± 0.6%.

The proportion of collagen fibre bundles in different regions of human periodontal ligament was studied by Gǫ̈tze and Kindler (1974), who compared the buccal, lingual, mesial and distal aspects of anterior and premolar teeth at different depths within the ligament. They found that although the volumetric proportions of collagen fibres are not depth dependent (Figure 4), there are more collagen fibres palatally

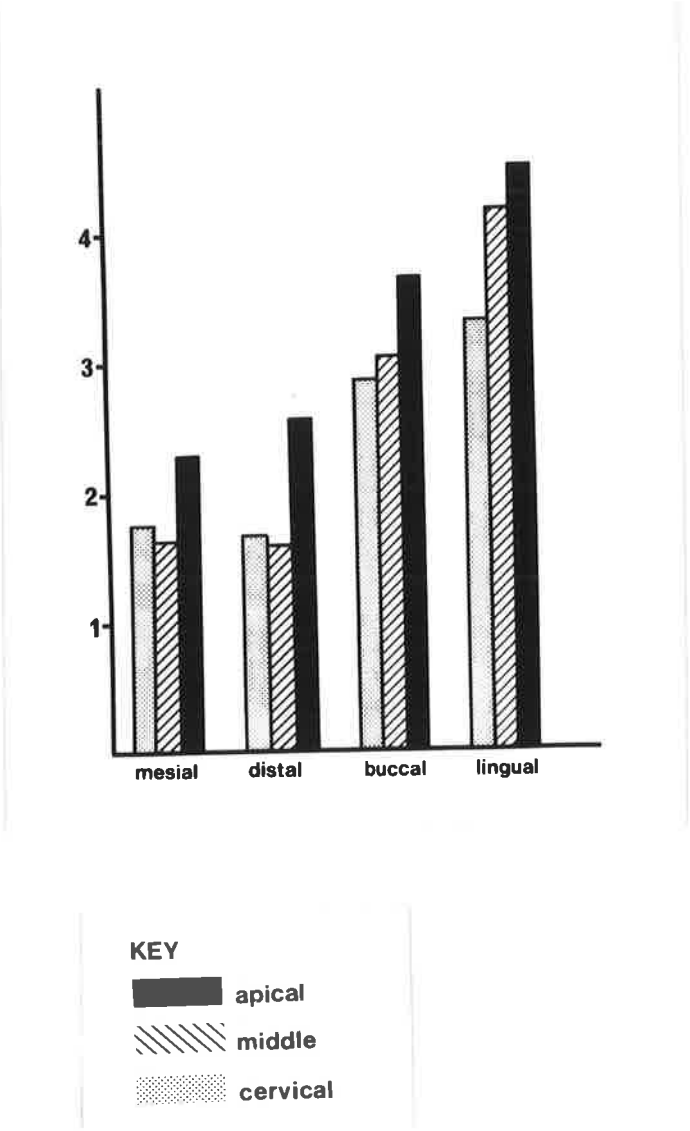


Figure 3: Variation in the volumetric proportion of human periodontal blood vessels in the ligament surrounding lower premolar teeth (Götze, 1976).

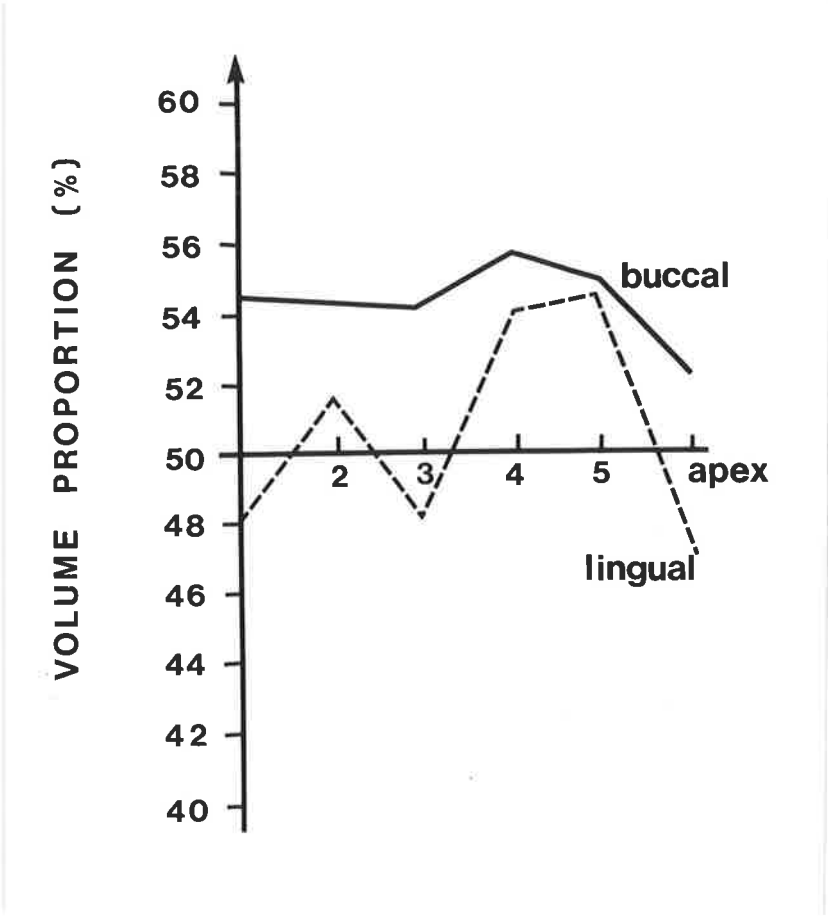


Figure 4: Variation in the volumetric proportion of human periodontal collagen with depth, in the ligament surrounding lower premolar teeth (Götze and Kindler, 1974).

than labially, and this difference is statistically significant in upper anterior and premolar ligament (Table 3).

| | | Lingual | | Labial | | Mesial | | Distal | |
|---|---|---|---|---|---|---|---|---|---|
| | N | F | I | F | I | F | I | F | I |
| Upper Ant. | 17 | 56.21 | 43.79 | 45.53 | 54.47 | 51.48 | 48.52 | 51.64 | 48.36 |
| Upper Prem. | 12 | 54.94 | 45.06 | 46.28 | 53.72 | 50.51 | 49.49 | 51.40 | 48.60 |
| Lower Ant. | 7 | 54.83 | 45.17 | 50.44 | 49.56 | 53.49 | 46.51 | 54.57 | 45.43 |
| Lower Prem. | 6 | 50.92 | 49.08 | 53.97 | 46.03 | 52.28 | 47.72 | 51.28 | 48.28 |

Table 3: Volumetric proportion of fibre bundles in different regions of human anterior and premolar teeth (from Götze and Kindler, 1974).

N - Number of teeth examined.

F - Volume % of fibre bundles.

I - Volume % of other components.

Biochemical quantification of periodontal collagen was undertaken by Guis, Shootweg and Tonino (1973) who estimated that nearly 50% of bovine periodontal protein is collagen. This compares favourably with the work of Götze and Kindler (1974).

In 1980, Götze confirmed his earlier findings stating that fibre bundles constitute 52 to 55% and blood vessels 2 to 2.8% of the ligament volume surrounding human upper anterior teeth.

The volumetric proportion of periodontal cells in mice and rats was investigated by Beertsen and Everts (1977), and Shore and Berkovitz (1979), respectively. These researchers estimated that between 42 and 60% of the extravascular volume of rat and mouse incisor periodontal ligament is comprised of cells.

Human premolar periodontal oxytalan fibres have been quantified using light microscopic techniques by Jonas and Riede (1980). These researchers reported that the volume proportion of oxytalan fibres increases from 3.03% to 4.97% following orthodontic treatment and that changes in other stereological parameters occur.

Quantification of the ligament surrounding normal and unimpeded rat incisors was undertaken using a TEM by Shore, Moxham and Berkovitz (1982). They reported that in extravascular periodontal tissue near the alveolar bone, oxytalan fibres have a volumetric proportion of 0.13% and an average of 1.20 ± 0.10 fibres in each 50 square microns of tissue. Changes in the number of fibres and their volumetric proportion are not statistically significant when the tooth is relieved from occlusion.

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 THE EXPERIMENTAL ANIMAL

A.L.C.A. strain mice, obtained from the Waite Agricultural Research Institute, Adelaide, South Australia, were kept in an animal house where temperature, humidity, and lighting were controlled. Distilled water and M. and V. mouse cubes were available in ample quantities until the animals were sacrificed at the age of 35 days.

## 4.2 ANAESTHESIA

All mice were anaesthetized by intraperitoneal injection of 30% Urethane solution (Appendix 8.1). This solution was administered at room temperature, at a dosage of 0.1ml/10g of body weight, using a 1.0ml disposable hypodermic syringe with a 26.5 gauge needle. When a response could not be elicited by squeezing the foot pad, anaesthesia was considered adequate.

## 4.3 ANTICOAGULANT

Following anaesthesia, heparinized saline (Appendix 8.2) was injected with a microlitre syringe into the ventral aspect of the tail vein, at room temperature, at a dose of 0.02ml/10g of body weight.

## 4.4 PERFUSION

Primary fixation was obtained by pulsatile intracardiac perfusion of glutaraldehyde and osmium tetroxide in 0.06M cacodylate

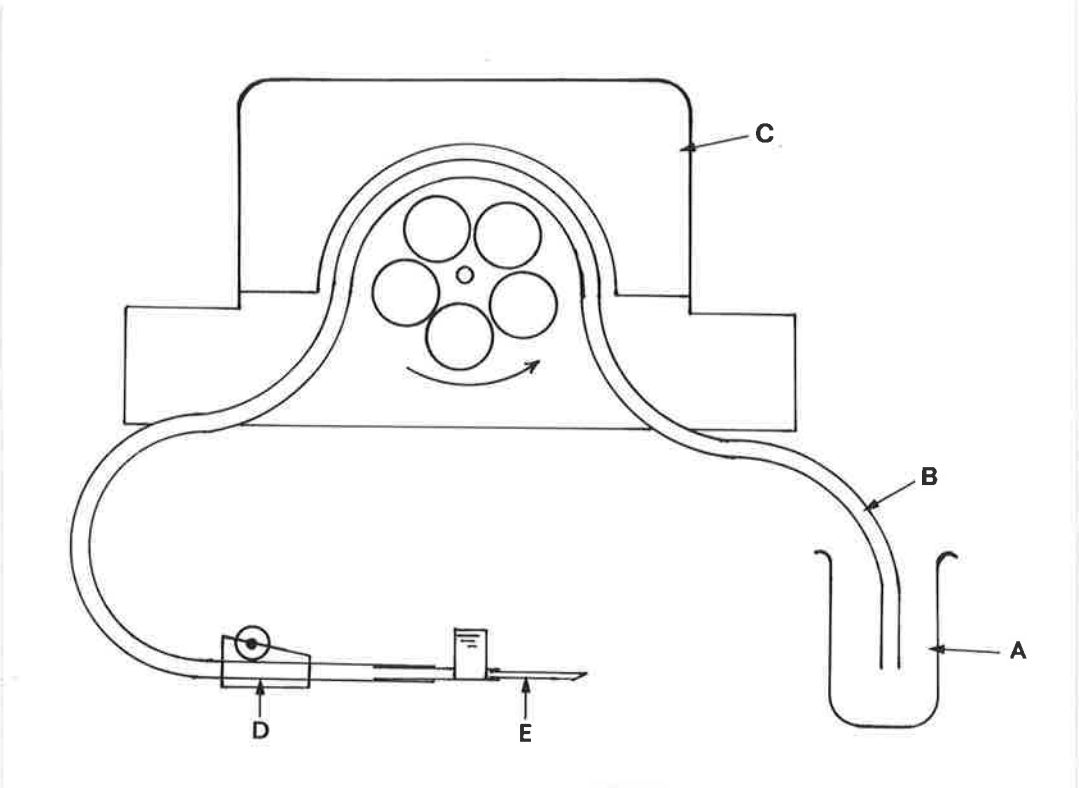


Figure 5: Schematic representation of the perfusion equipment. (Aron alpha was used to seal all joints.)

A - 50 ml Glass beaker.
B - Silastic tubing.*
C - Peristaltic pump.
D - Flow regulator.
E - Butterfly needle 25G.

* Inner diameter: 1.57mm. Outer diameter: 2.41mm.

buffer (Appendix 8.6). Once narcotized, the anticoagulated mouse was strapped with leukosilk sticking plaster to a wire gauze platform resting in, and protruding above, a large petri dish.

A sub-dermal incision, made below the xiphisternum, was extended in the midline anteriorly to the clavicles. Lateral relieving incisions allowed the dermal tissues to be reflected back and the rib cage exposed. The anterior portion of the rib cage was then removed and left ventricular cannulation achieved before the cessation of respiration. Whilst the heart was still beating the peristaltic pump was started and the right atrium incised. The perfusate was delivered at a rate of 2.5ml/minute using diastolic and systolic pressures of 80 and 100mmHg respectively, and a pulse rate of 60 beats/minute. In accordance with Thorball and Tranum-Jensen (1983), the flow rate was increased gradually by adjustment of a flow regulator (Figure 5), and the perfusate was introduced for at least five minutes to saturate tissues.

## 4.5 DISSECTION

Following successful perfusion, the animals were immediately decapitated. The mandibles were then dissected free from the surrounding tissue and kept moist in 0.06M cacodylate buffer (Appendix 8.4) whilst sectioned in the midline and cleaned with a scalpel blade.

## 4.6 DEMINERALIZATION

Once trimmed, hemisectioned mandibles were placed into labelled histokinette baskets and demineralized at 4°C in a 600 ml glass beaker containing EDTA solution at pH 6 (Appendix 8.7). This solution was

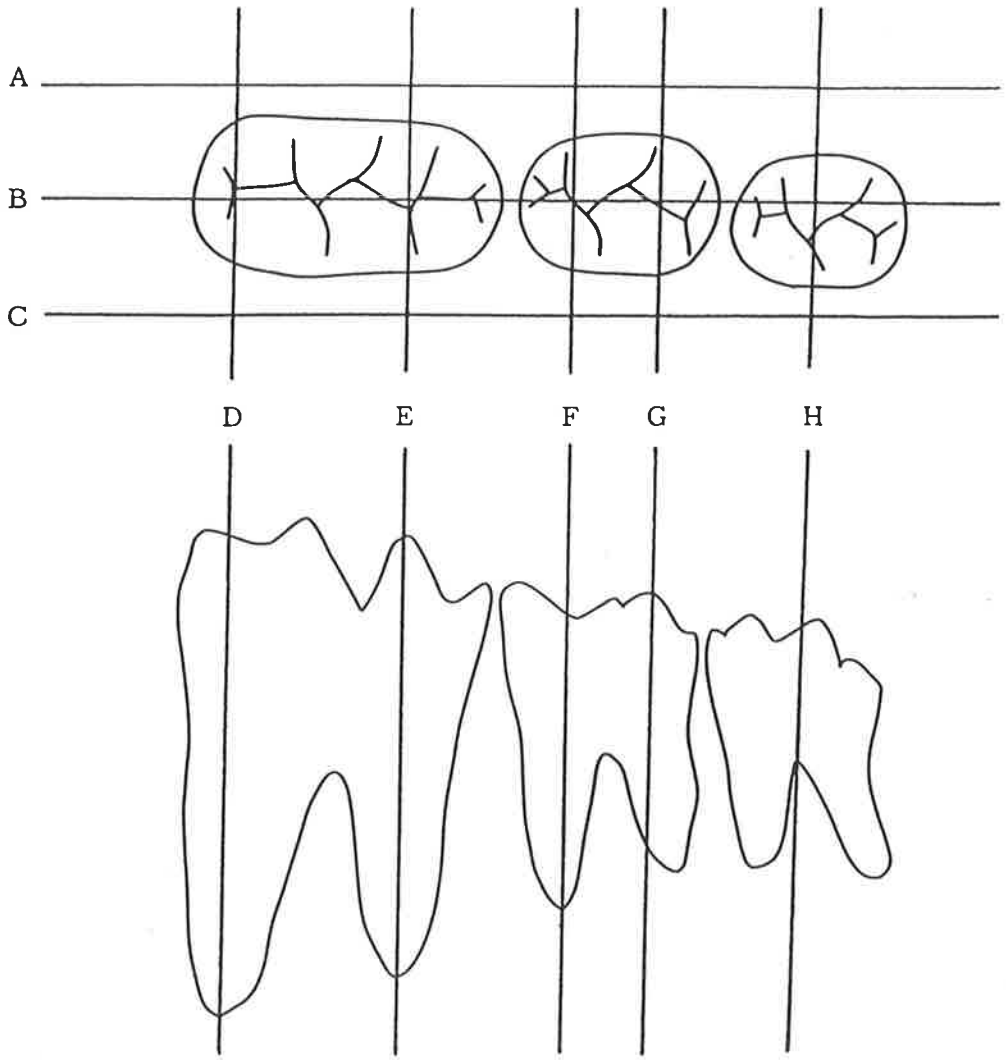


Figure 6: The sequence used for trimming the right side of the mandible. The trimming sequence for the left side of the mandible is a mirror image of that illustrated above.

M1 - 1st mandibular molar.
M2 - 2nd mandibular molar.
M3 - 3rd mandibular molar.

A to H represent the sequence of incisions used to obtain tissue blocks.

continuously agitated, and changed daily. The end point of decalcification was determined to be 21 days, by using dental radiographic equipment (Appendix 8.12) and probing the tissue with a fine needle.

## 4.7 TRIMMING

Following decalcification the tissue was embedded in Xantopren heavy bodied impression material contained in wax moulds. The mandibles were sectioned using a double edged razor blade which was discarded after each incision. The use of a stereo-dissecting microscope facilitated trimming, and a diagram was drawn of each section to enable orientation during embedding. Dehydration was avoided by bathing the tissue continuously with chilled 0.06M cacodylate buffer. Tissue segments were then transferred to separate, metal capped 2ml soda glass vials half filled with 0.06M cacodylate buffer.

## 4.8 TISSUE PROCESSING

During processing, tissue portions were continuously rotated in separate vials and solutions were changed at room temperature with soda glass Pasteur pipettes. The following tissue processing regime was adhered to.

(i) Wash

Immediately after trimming tissue was washed overnight in 0.06M cacodylate buffer (Appendix 8.4).

(ii) Post-fixation

4% osmium tetroxide in double distilled water for one hour (Appendix 8.5).

(iii) Wash

0.06M cacodylate buffer for 15 minutes (Appendix 8.4).

(iv) Wash

70% alcohol for 15 minutes.

(v) Block stain

1% uranyl nitrate in 70% alcohol for 1 hour (Appendix 8.8).

(vi) Wash

70% alcohol for 15 minutes.

(vii) Dehydration

2 x 15 minutes in 70% alcohol.

2 x 15 minutes in 80% alcohol.

2 x 15 minutes in 90% alcohol.

2 X 15 minutes in 100% anhydrous alcohol.

2 x 15 minutes in propylene oxide.

2 x 30 minutes in propylene oxide.

All diluted alcohols were prepared with double distilled water.

(viii) Infiltration

12 hours in 1:1 (propylene oxide : LX-112).

12 hours in 1:3 (propylene oxide : LX-112).

12 hours in LX-112 embedding resin (Appendix 8.9).

## 4.9 EMBEDDING

Fresh LX-112 prepared for final embedding was allowed to stand overnight. Using a stereomicroscope, tissue samples were removed from their vials and then placed with known orientation into silicone rubber moulds. The resin filled moulds were incubated at 37°C for 48 hours, and then at 60°C for a further 48 hours before being coded and stored at room temperature.

## 4.10 SAMPLE SELECTION

Tissue blocks from different sides of two animals were selected by lottery to form a pilot study. These blocks were sectioned perpendicular to the long axis of the tooth. Sections in the silver interference range were collected from 21 levels, spaced 50 microns apart, from the alveolar crest to the tooth apex. To complete the study, tissue from the opposite sides of the original two animals, and from the left and right molars of another randomly selected animal, were examined. The four blocks that comprised the latter part of the study were sectioned in a similar manner to those in the pilot study, but silver sections were collected at only 6 levels, spaced 200 microns apart.

## 4.11 SECTIONING

Each block was reduced in size, secured in Reichert specimen holder for flat embedding, and mounted on a Reichert-Jung Om-U4 ultramicrotome. A no. 11 scalpel blade was used to trim the block face to a divergent sided trapezoidal mesa and to complete other initial trimming. The block and chuck were then transferred to the specimen arm of the ultramicrotome and a glass knife aligned in the knife holder. Glass knives were prepared using a LKB Type 780 1B knifemaker.

One micron thick orientation sections were transferred, using a wire loop, to a pool of millipored, double-distilled water on a clean glass microscope slide. These sections were then flattened on to the slide atop a 70°C hotplate, and stained for two minutes at 70°C with millipored solutions of 0.05% toluidine blue and 1% borax (Appendix 8.10). Sections were then rinsed with millipored double

distilled water, differentiated in 50% ethanol for 30 seconds, and dried on the hotplate. The tissue was examined using an Olympus EHT light microscope and photographed using an Olympus camera and Polaroid Type 107 black and white Land film as required. Because periodontal disease has not been reported in mice of this age, the first section exhibiting a complete band of alveolar bone was considered to represent the alveolar crest, and this was used as a reference marker for later depth measurements.

At this point the mesa face was trimmed to include alveolar bone, periodontal ligament, and adjacent tooth. Silver sections, approximately 70 nm thick, and containing the most mesial aspect of the ligament were cut, using a Diatome diamond knife, with a clearance angle of 10° and a cutting speed of 1mm/second. These sections were floated on a bath of millipored, double-distilled water, flattened with chloroform vapour and placed on to clean, uncoated, 3mm diameter, VECO 200 mesh copper grids. Sections were collected by holding the copper grid with its dull surface towards the water bath, slowly lowering the grid to contact the sections and then lifting the grid and attached sections perpendicularly from the water surface. Grids were then dried face upwards on filter paper in a covered petri dish and stored in a LKB specimen grid holder.

Further 1 micron thick sections were collected until the next depth to be examined was reached. At this point an orientation section was collected, mounted on a glass slide, stained, and photographed. The mesa face was trimmed, and silver sections placed on cleaned 200 mesh copper grids. This procedure was repeated at predetermined intervals (section 4.10), until the tooth apex was reached.

## 4.12 GRID STAINING

Dried grids were first stained, tissue side down, for 8 minutes on a droplet of 0.5% uranyl acetate (Appendix 8.11.(i)) maintained at 37°C. This was done by placing grids in microfiltered droplets of freshly prepared stain on Parafilm 'M' laboratory film, in a covered petri dish, that had been preheated on a thermostatically controlled hotplate. Grids were then rinsed by agitating them vigorously for 30 seconds in each of four beakers containing 100ml millipored, double-distilled water at 37°C.

Grids were then stained with lead citrate for 4 minutes by floating the grids in a microfiltered droplet of freshly prepared modified Reynold's lead (Appendix 8.11.(ii)), on a square of Parafilm 'M' laboratory film in a covered petri dish. Also enclosed in this petri dish were sodium hydroxide pellets to absorb contained carbon dioxide and minimize the formation of lead carbonate precipitate on tissue sections.

The tissue was again rinsed in freshly double-distilled, millipored water and dried, tissue surface uppermost, on fine grade filter paper. Grids were then stored in a LKB specimen grid holder until required for transmission electron microscopy.

## 4.13 TRANSMISSION ELECTRON MICROSCOPY

A Jeol 100S transmission electron microscope (Appendix 8.13) was used to examine tissue sections. Grids were placed tissue side downwards in the vaccuum column of the microscope and conditioned in the electron beam before being examined.

## 4.14 MICROGRAPHY

Ultramicrographs were obtained with Ilford Electron Microscope Film using the inbuilt photographic equipment of the Jeol 100S. This equipment has an automatic aperture setting and an exposure time of 4 seconds was used. Focussing was assisted by use of an image wobbler which was switched off immediately prior to micrograph exposure. The width of the ligament was trisected and one micrograph was taken at a magnification of 3,000x in each section. Areas photographed for quantitative analysis were selected on the basis of unaligned, fixed orientation systematic, quadrat subsampling (section 6.1.e). This procedure maximized tissue support and micrograph randomness. A picture of a replicating graticule at a magnification of 3,000x was also exposed at this stage. In the pilot study, three further ultramicrographs, at a magnification of 10,000x, were taken from each third of the ligament to aid with tissue identification. In the second part of the study, features of special interest were also photographed.

## 4.15 DEVELOPING AND PRINTING

Ultramicrographs were agitated in Kodak D19 Developer for 4 minutes at 20°C, rinsed in running water and fixed in Hypam Rapid Fixer at 20°C for 8 minutes. Following fixation, negatives were again washed under running water for 15 minutes and rinsed in a mixture of wetting agent and deionized water. After drying, negatives were identified and stored in cellophane envelopes.

All micrographs to be used for stereological analysis were printed at a final magnification of 8,500x using a Durst Laborator 54 enlarger. Replicating graticules were used to ensure that variation in

magnification between micrographs was eliminated. Ilfospeed photographic paper, processed with Ilfospeed paper developer and fixed in Hypam rapid fixer, was used for printing.

## 4.16 POINT COUNTING PROCEDURE

Each photomicrograph was masked to leave an area of 85 x 114mm uncovered as this corresponded to an area of 20 x 28 microns of periodontal ligament. To each micrograph two test grids were applied. Firstly, a 35 point square lattice grid, with adjacent points spaced 34mm apart was placed over the photograph and profiles underlying each point scored in accordance with the categories listed in Appendix 8.14. Subsequently, a 140 point square lattice grid, with adjacent points spaced 17mm apart, was applied to each photomicrograph and points overlying profiles of special interest counted. In those instances when a point of the test system lay on a boundary between two profiles, the profile above and to the right of the test point was scored. Finally, the number of profiles of cells, blood vessels, nerve axons and oxytalan fibres on each photomicrograph were counted using the convention of forbidden lines (section 6.1.f) and the data tabulated for morphometric and stereological analysis.

## 4.17 STATISTICAL ANALYSIS

Data from both the pilot and extended study was initially grouped into the categories listed in section 5.3.a, and then analysed using routines in the BMDP-81 statistics package. Description of the statistical tests used are presented with the appropriate findings. Relevant stereological equations are presented in Appendix 8.15.

# CHAPTER 5

# RESULTS OF THE INVESTIGATION

## 5.1 EVALUATION OF TISSUE PREPARATION

### a. FIXATION

Macroscopically, successful perfusion with osmium tetroxide and glutaraldehyde caused the animals to become black and rigid. Discolouration of abdominal and thoracic organs, foot pads, neck musculature, nose tip, palate, tongue and mandibular canal were indicative of optimal periodontal perfusion.

Light microscopically, the tissue appeared well preserved and free from distortion. Red blood cells were retained in few vascular channels.

Ultrastructurally, the integrity of mitochondrial membranes remained intact in all regions of the ligament. Some small pockets of degenerative cellular material 1 to 2 microns in diameter occurred within the ligament. These regions were most frequently perivascular in location and it was not ascertained if this represented normal cell death or lack of fixative penetration. Occasional myelin bodies were the only other fixation artifacts present within the tissue.

### b. TISSUE STAINING

Excellent contrast distinctiveness of tissue components was obtained using the present uranyl acetate and lead citrate staining method. The formation of lead precipitate on tissue sections was an

infrequent occurrence when sodium hydroxide pellets were changed frequently during staining with Reynold's lead.

## 5.2 MORPHOLOGICAL FINDINGS

### a. FIBROBLASTS

Fibroblasts were pleomorphic cells which occurred abundantly throughout the body of the ligament and were generally orientated radially to the tooth in transverse section between the principal collagen fibres. In the vicinity of nerves and blood vessels these cells were more randomly orientated to the tooth surface than elsewhere in the ligament. Ultrastructural features characterizing fibroblasts were:

(i) abundant rough endoplasmic reticulum, arranged in parallel array within the cytoplasm;

(ii) few free ribosomes and polyribosomes arranged as rosettes;

(iii) a prominent, ovoid nucleus, with peripheral condensations of heterochromatin;

(iv) a Golgi apparatus that was most frequently located in the perinuclear region; and,

(v) numerous cytoplasmic microfilaments.

Pinocytotic type vesicles were a relatively common ultrastructural finding as were slender cell processes, up to 5 microns in length, that made numerous membranous contacts with adjacent cells. However, specialized cell junctions were not found between cells.

When present, intracellular collagen profiles occurred in the peripheral cytoplasm, and were orientated parallel to adjacent extracellular fibres. Oxytalan fibres occurred within invaginations at

cell surfaces and, occasionally, were completely surrounded by fibroblast processes.

Although fibroblast profiles were usually mononuclear, some multinucleated cells were also seen. However, these multinucleated cells may have been an artifact of the plane of section as multiple centrioles were not found in these cells.

Ciliated fibroblasts were an uncommon finding and occurred most frequently in the alveolar third of the ligament. The cilia often lay parallel to the cell surface with the end of the cilium tucked into an invagination in the cell membrane (Figure 7). These organelles had a 9 + 0 arrangement of microtubules and some fibroblasts contained more than one cilium.

Fibroblast-like cells, characterized by flat, cytoplasmic sheets containing short, dilated profiles of rough endoplasmic reticulum and few mitochondria, formed an incomplete investing layer around some blood vessels. Vesicles and invaginations were frequently located at the cell surface and, more commonly, on the surface nearest the vessel lumen. Occasionally, microfibrils were seen to interrupt the continuity of these cells, which were considered to be veil cells. Cells, with a similar morphology to veil cells, formed an investing sheath around periodontal nerves. Other cells, that contain an ovoid nucleus and a reduced amount of cytoplasm, were also found, predominantly in the perivascular regions of the periodontal ligament. It was considered that these cells may represent progenitor cells for the fibroblast population.

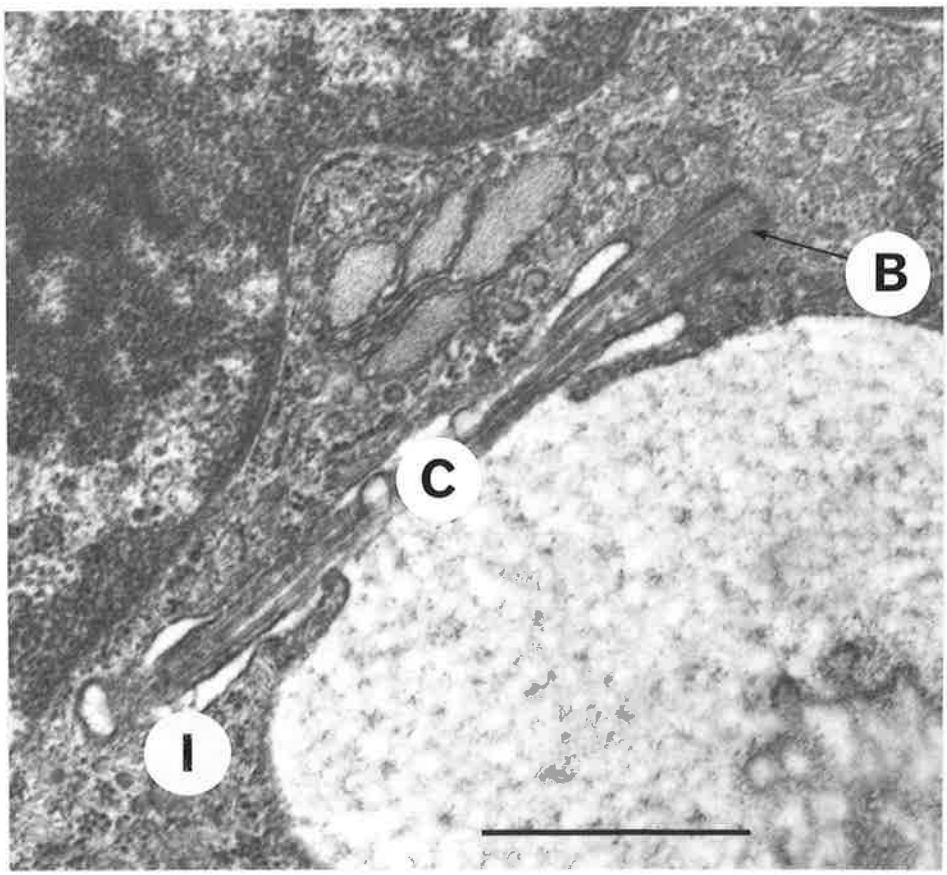


Figure 7: High power view of a fibroblast cilium (C) with a length of 2.3 microns tucked into a cell invagination (I). Although the microtubule arrangement cannot be determined from this section, the basal body (B), from which the cilium arises, is evident.

Region: Depth 700 microns, alveolar third.

Magnification = 37,500x. Bar = 1 micron.

b. OSTEOBLASTS

As a result of the plane of section and the orientation of cells, difficulty in differentiating osteoblasts from fibroblasts was encountered. However, cells morphologically similar to fibroblasts, but located adjacent to the socket wall, were considered to be osteoblastic in function. These cells occasionally extended cytoplasmic processes into the alveolar bone and had adjacent "satellite" cells situated near the osteoblast nucleus (Figure 8).

c. CEMENTOBLASTS

Similar difficulty was encountered in differentiating cementoblasts from fibroblasts situated near the tooth surface. However, four morphologically dissimilar types of cementoblast-like cells were seen. Firstly, in the apical half of the ligament, cementoblasts which were trapezoidal or cuboidal in cross-section, were situated with their broadest base subjacent to the cementum surface and small pseudopodal extensions from these cells extended towards the cementum. Interspersed between the base of these cells and the cementum was a fine collagenous matrix 0.2 to 0.5 microns wide. Secondly, cells with a similar ultrastructure and juxtaposition to the tooth root as the first cell type, but without cell processes extending towards cementum, occurred in regions of acellular cementum. Thirdly, in the apical half of the root, some cementoblasts were located up to 5 microns from the tooth surface. These cells had fine cytoplasmic processes that extended towards and, occasionally, inserted into cementum and were separated from the root by collagen bundles that ran parallel to the tooth surface. Fourthly, a smaller cell type, with few cytoplasmic processes, was present 1 to 3 microns from the

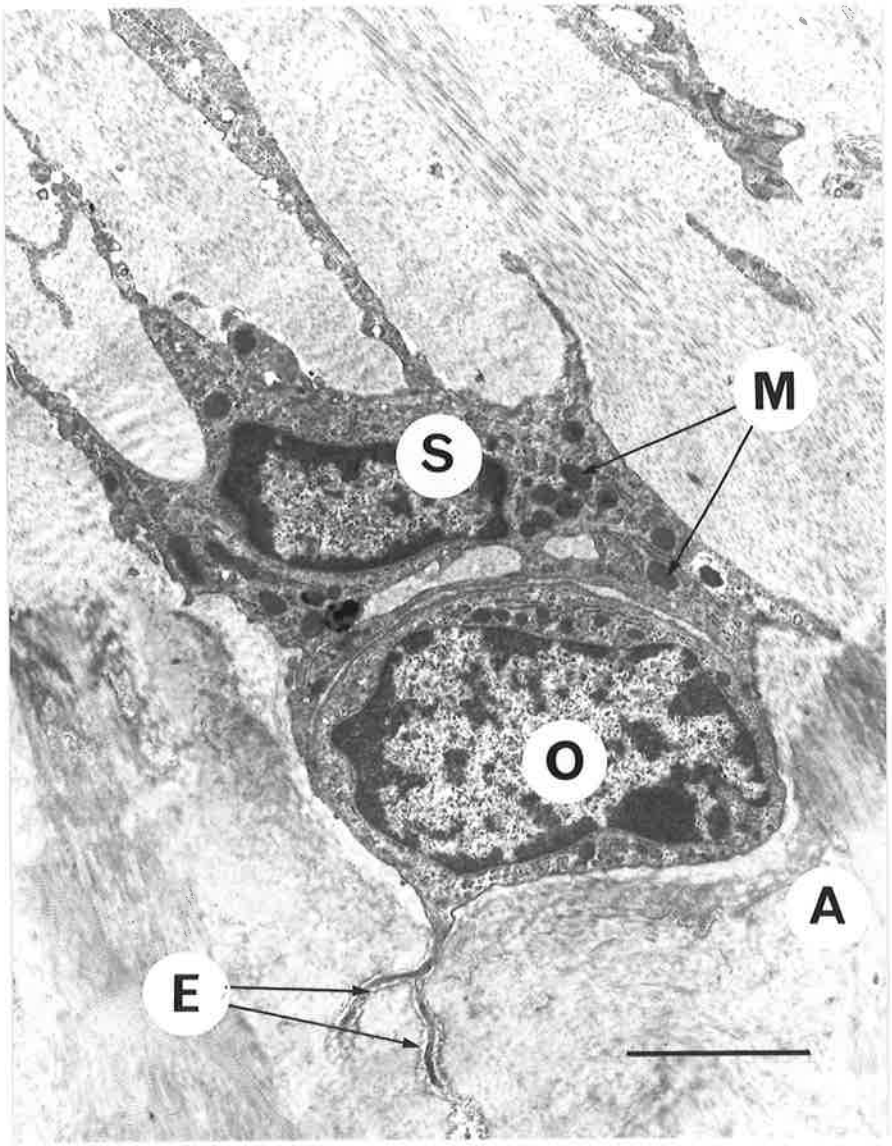


Figure 8: A presumptive osteoblast with a reduced amount of cytoplasm (O) being incorporated in, or emerging from, alveolar bone (A). Fine cytoplasmic extensions (E) penetrating the alveolar bone branch and presumably contact underlying osteocytes. A small "satellite cell" (S), with numerous mitochondria (M), frequently occurred adjacent to these cells.

Region: Depth 250 microns, alveolar third.

Magnification = 11,250x. Bar = 2 microns.

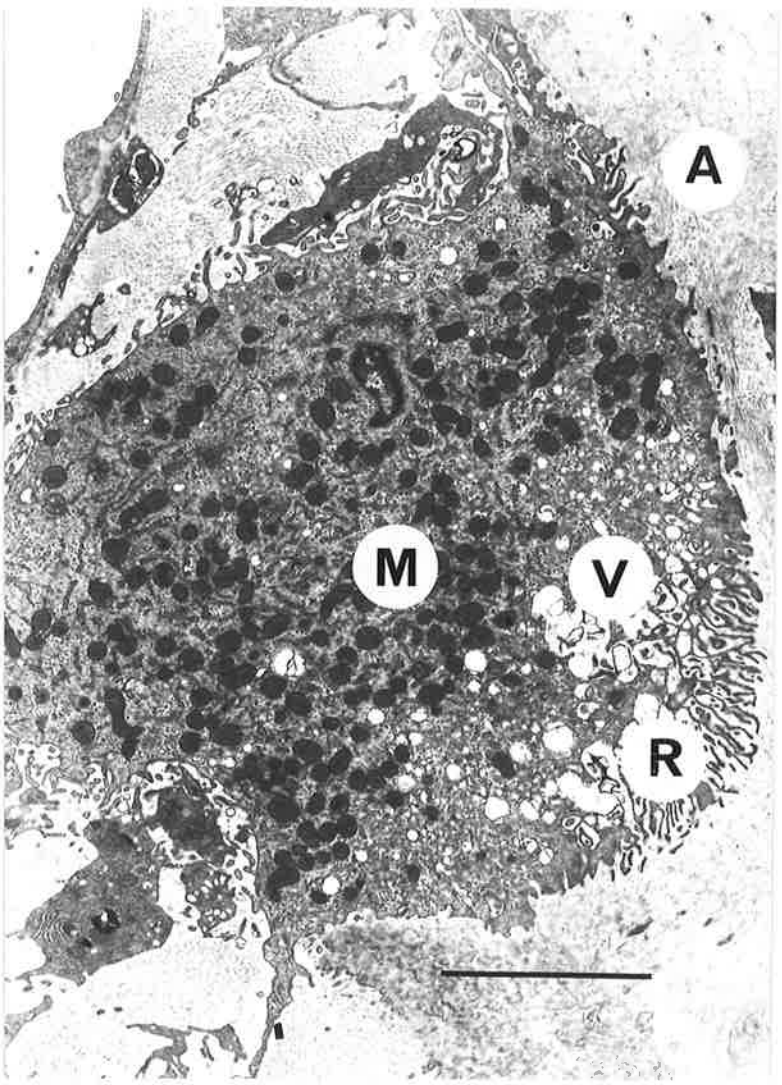


Figure 9: An osteoclast lying adjacent to alveolar bone (A), partially enclosed in a Howship's lacunae. Characteristic features of these cells include their size, the presence of a ruffled border (R), numerous vacuoles (V), and mitochondria (M) located subjacent to this vacuolar region. Although multinucleated, no nuclei are present on this section.

Region: Depth 750 microns, alveolar third.

Magnification = 5,500x. Bar = 5 microns.

tooth surface. These cells, which contained few synthetic organelles and appeared to be metabolically inactive, were found along the length of the root and were considered to be precursor cells for either cementoblasts or fibroblasts.

d. OSTEOCLASTS

Osteoclasts within the ligament were large cuboidal or columnar cells, up to 25 microns in diameter, containing numerous cell nuclei which congregated at the end of the cell furthest from the bone surface. A distinct ruffled border was apparent in those sections that passed through the central portion of the cell and, adjacent to this border, many vacuoles with a diameter of up to 1 micron in diameter were present. Mitochondria, located between the vacuolar layer and the cell nuclei, were elongated or ovoid in cross-section, and larger than those seen in fibroblasts. Ribosomes were arranged as short profiles of rough endoplasmic reticulum, or as rosettes throughout the cell.

e. CEMENTOCLASTS

Multinucleated cells were not seen at the cementum surface in these 35 day old mice.

f. EPITHELIAL CELLS

Epithelial cells varied according to their position within the periodontal ligament. They occurred as groups of 3 to 4 cells in close apposition to the root surface, but separated from it by a narrow band of loose collagenous material up to 0.5 microns in width. These cells were found at depths greater than 400 microns, and were easily identified because of:

(i) the paucity of rough endoplasmic reticulum;

(ii) relative prominence of polyribosomes and mitochondria; and,

(iii) the presence of a basement lamina, tonofilament bundles, tight junctions and interdigitating microvillus extensions (Figure 10).

Whereas some epithelial cells appeared to have undergone degenerative changes (Figure 11), others appeared to be incorporated into the forming cementum (Figure 12).

g. PERICYTES

Pericytes were elongate cells with little cytoplasmic branching and a basement lamina that was continuous with that of the endothelium. These cells were located in the walls of some periodontal vessels, more commonly around vessels with a lumenal diameter of 4 to 8 microns. In vessels with a lumenal diameter greater than 20 microns this cellular investment was usually absent or incomplete. Ultrastructurally, pericyte nuclei were flattened and rough endoplasmic reticulum and mitochondria were sparse. Ribosomes usually occurred singularly, or as rosettes, and peripheral vesicles within these cells most frequently occurred at the cell surface adjacent to the vessel lumen. Adjacent pericytes rarely contacted one another.

h. SCHWANN CELLS

Ensheathing cells of myelinated and non-myelinated nerves were found within the periodontal ligament. The bodies of these cells were not common. However, the characteristic coiled myelinated sheath around myelinated nerves, and the fine cytoplasmic extensions around

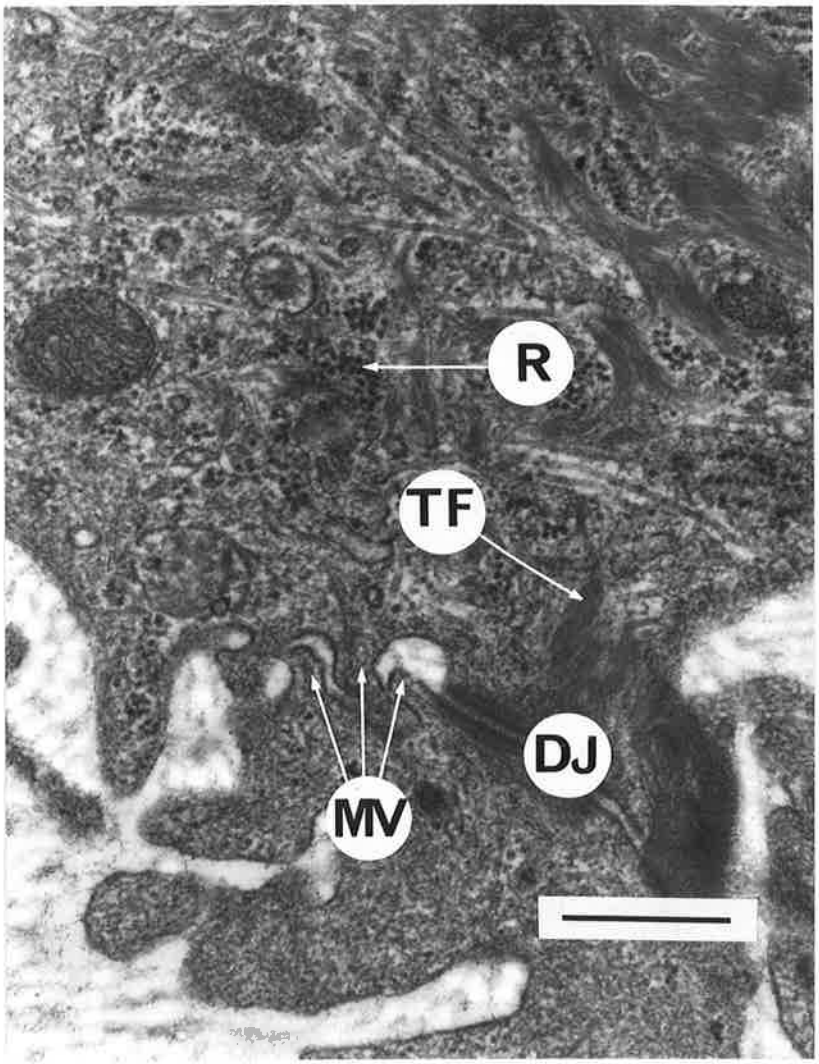


Figure 10: Micrograph illustrating characteristic features of epithelial cells including desmosome junctions (DJ) with associated tonofilament bundles (TF), interdigitating microvillus extensions (MV), and ribosomes (R). A basement membrane and prominent Golgi apparatus are not evident on this micrograph.

Region: Depth 400 microns, tooth third.

Magnification = 45,000x. Bar = 0.5 microns.

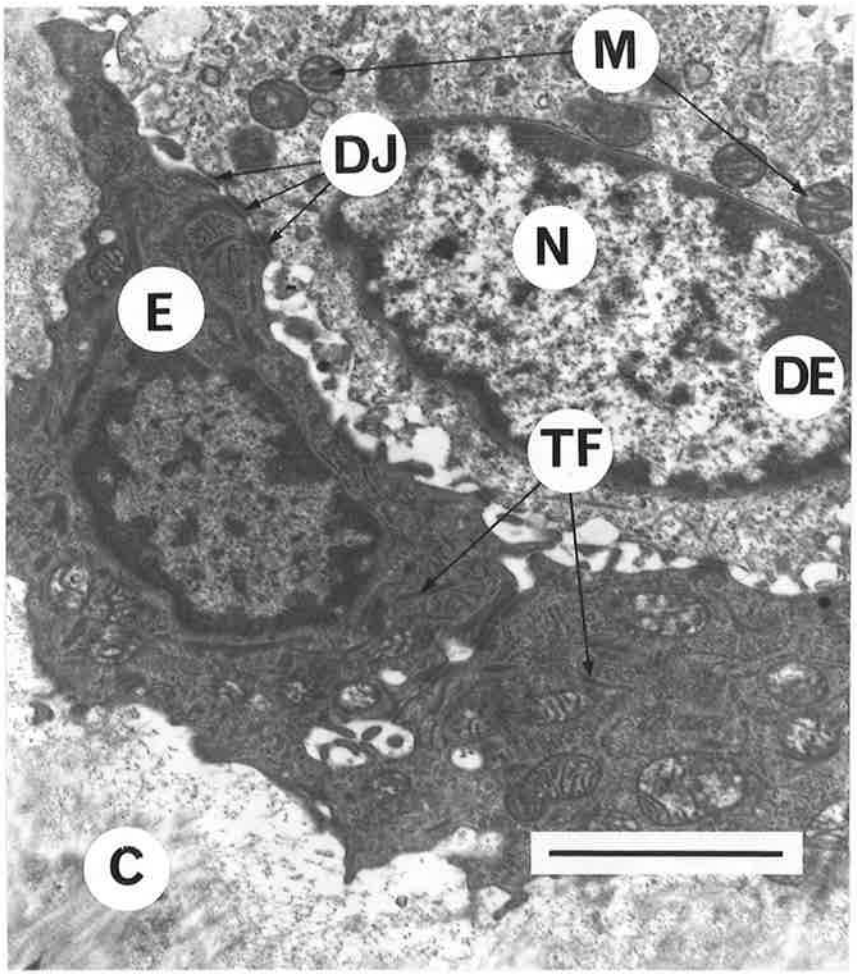


Figure 11: A mature epithelial cell (E) situated adjacent to the cementum surface (C). In the degenerating epithelial cell (DE), mitochondria (M) and desmosomes junctions (DJ) are numerous. However, the nucleus (N) has become less densely staining and tonofilament bundles (TF), present in the mature epithelial cell, have disappeared.

Region: Depth 750 microns, tooth third.

Magnification = 15,000x. Bar = 2 microns.

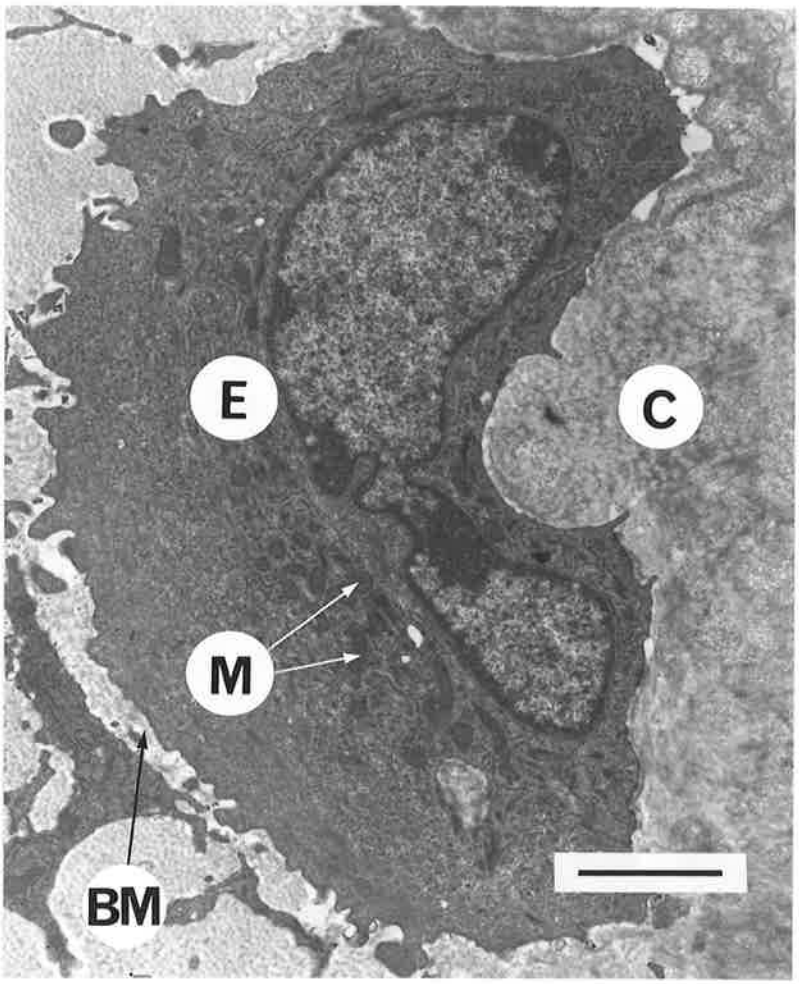


Figure 12: An epithelial cell (E) being incorporated into adjacent cementum (C). Note that the basement membrane (BM) is tenuous. Numerous mitochondria (M) are located in the perinuclear region.

Region: Depth 650 microns, tooth third.

Magnification = 10,000x. Bar = 2 microns.

unmyelinated nerves, were commonplace. Ultrastructurally, Schwann cells were surrounded by a basement membrane and contained few mitochondria or profiles of rough endoplasmic reticulum. Membrane bound vesicles, and associated cell invaginations, were present at the cell surface on the connective tissue aspect of some cells.

i. K-CELLS

Rounded cells, surrounded by a basement membrane, that contained a kidney-shaped nucleus with peripheral condensations of heterochromatin, were found within the mouse molar periodontal ligament. Extensions of rough endoplasmic reticulum with dilated cisternae, mitochondria, and a prominent Golgi apparatus, were also prominent features (Figure 13). Furthermore, these cells contained numerous peripheral vesicles, with a diameter of less than 100 nm, that were located within the cell body and in cell processes that extended to enclose adjacent unmyelinated nerves (Figure 14).

These cells were found only in the apical half of the ligament, most frequently between the depths of 600 and 800 microns. Although present in the body of the ligament, K-cells most commonly occurred near blood vessel lumen.

j. MACROPHAGES

Macrophages could be differentiated from periodontal fibroblasts by the presence of auto- and heterolysosomes. Ribosomes occurred predominantly as polyribosomes, rather than rough endoplasmic reticulum. Cylindrical cytoplasmic extensions up to 1.5 microns in

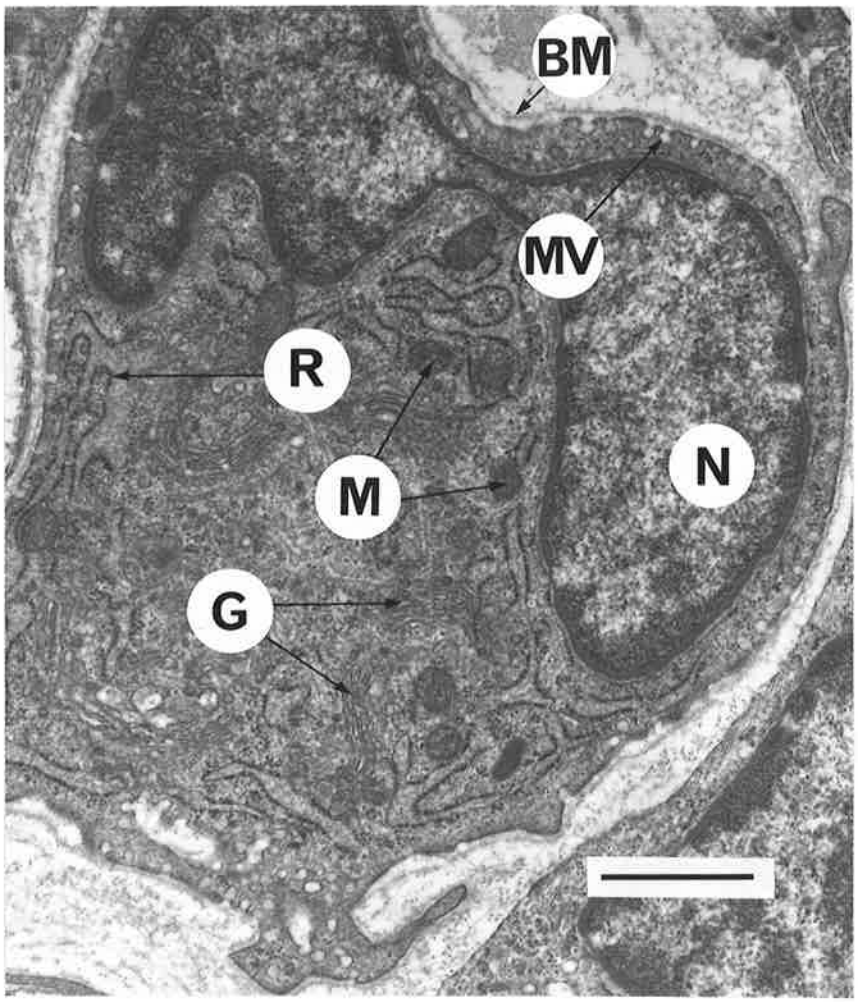


Figure 13: High power view of the ultrastructure of a K-cell. A kidney shaped nucleus (N), extensive Golgi apparatus (G), mitochondria (M), and rough endoplasmic reticulum (R) are characteristic of these cells. Peripherally, a basement membrane (BM) and microvesicles (MV) are present. Leptomeric organelles, described in the current literature, were not found in these cells with the magnifications used.

Region: Depth 700 microns, alveolar third.

Magnification = 20,000x. Bar = 1 micron.

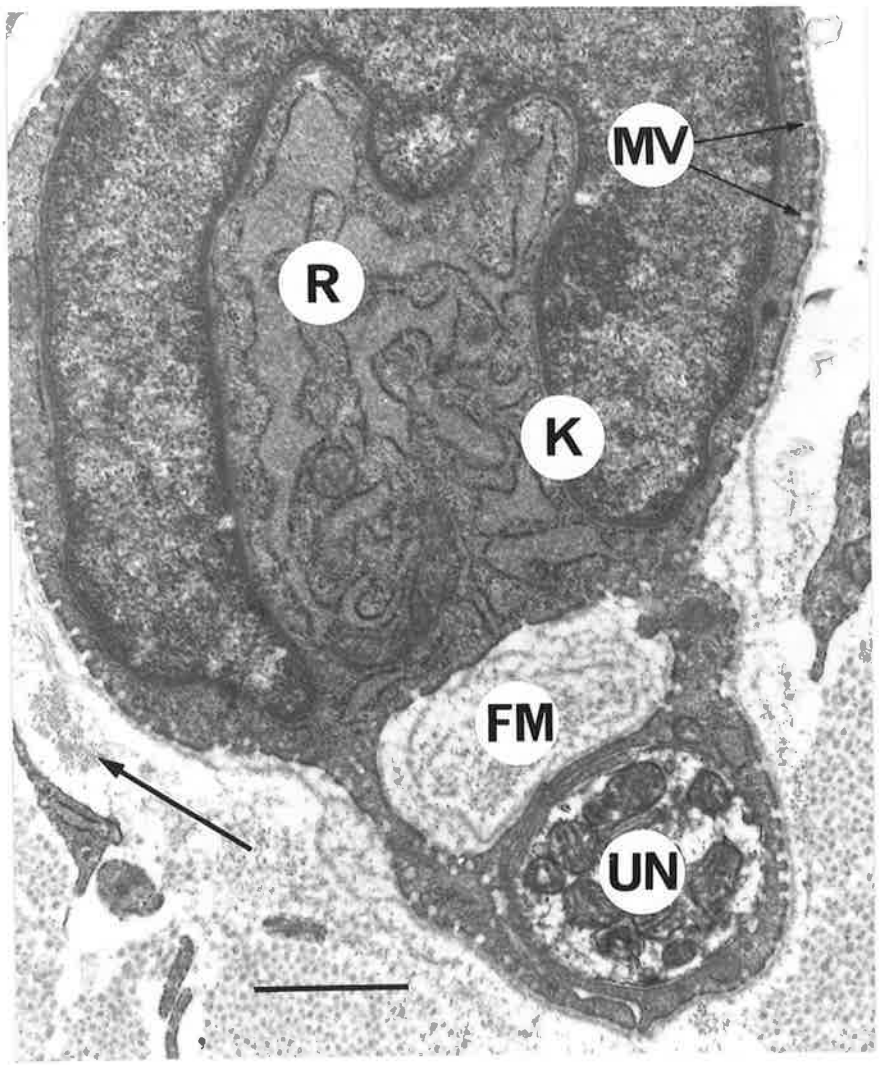


Figure 14: The characteristic juxtaposition of K-cells (K) to mitochondria rich unmyelinated nerves (UN), with a fine fibrillar matrix (FM) enclosed between these two structures. This micrograph also illustrates the large number of peripheral microvesicles (MV) and rough endoplasmic reticulum with dilated cisternae (R). An oxytalan bundle (arrowed) lies adjacent to the cell.

Region: Depth 650 microns, alveolar third.

Magnification = 20,000x. Bar = 1 micron.

length, frequently orientated parallel to the cell surface, were also a feature of these cells.

k. DEGENERATING CELLS

Cells with few synthetic organelles, cytoplasmic vacuolization, ruptured cell membranes, and a loss of cell structure, were seen within the periodontal ligament. These cells were frequently perivascular in location.

l. COLLAGEN FIBRES

Principal fibres were orientated radially to the tooth surface in transverse section, except in the regions of blood vessels and nerves where collagen fibres appeared to be finer and more randomly arranged. Collagen fibres had a tortuous course, and thus appeared in longitudinal and transverse section within the same fibre bundle.

m. OXYTALAN FIBRES

Oxytalan fibres ran perpendicularly to the plane of section being obliquely orientated to the collagen fibres within the ligament. The oxytalan fibres were situated near nerves and located within cell invaginations of both fibroblasts and macrophages. Oxytalan fibres were also found completely enclosed by collagen bundles, as well as adjacent to endothelial cells in blood vessel walls. Fibres were comprised of numerous, fine microfibrils that were orientated parallel to the oxytalan fibre bundle and interspersed with an amorphous interfibrillar material.

n. CEMENTICLES

In the apical half of the periodontal ligament, circular structures 1 to 4 microns in diameter were apparent up to 6 microns from the cementum surface. These structures, located in close proximity to cementoblasts and epithelial cells, consisted of aggregations of fibrillar material that was similar in appearance to decalcified cementum matrix. Thus, these structures resembled a preliminary calcifying front, distant to the completely mineralized cemental surface.

o. BLOOD VESSELS

Periodontal blood vessels were most frequently orientated perpendicularly to the plane of section and located near the socket wall. Four morphologically distinct types of vessel present within the ligament were:

(i) capillary sized vessels with a partial or complete pericytic cellular investment;

(ii) capillary sized vessels without a pericytic cellular investment;

(iii) postcapillary sized venules with associated pericytes being few in number, or absent; and,

(iv) postcapillary sized vessels with a complete pericytic cellular investment.

The endothelial lining of capillary sized vessels varied in width from 0.2 microns, to 1.5 microns in the region of the endothelial cell nucleus. The nucleus either bulged into the vessel lumen or into the surrounding connective tissue. Up to six endothelial cells formed

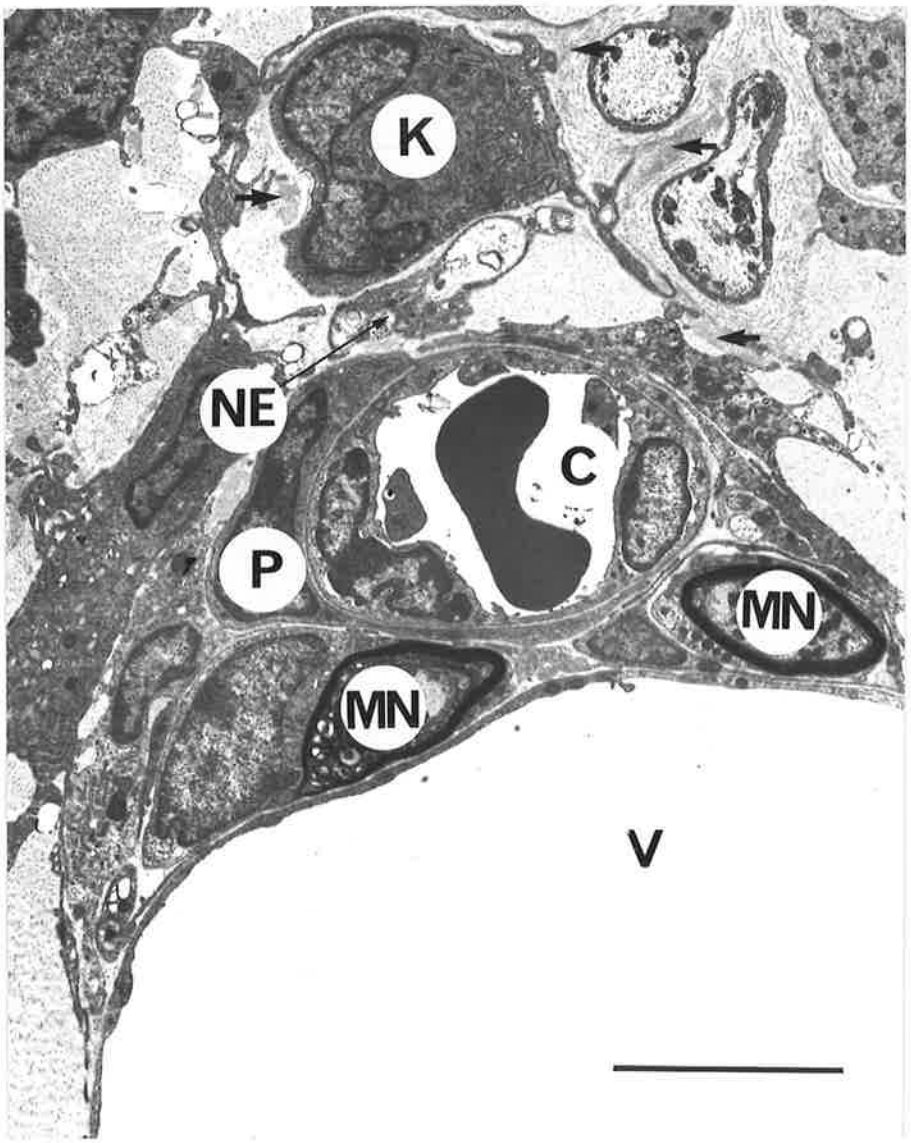


Figure 15: A capillary-sized vessel (C) with a presumptive pericyte (P) situated in the vessel wall. This vessel lies in close proximity to a larger venous vessel (V), and from the work of Wong (1983) it might be anticipated that these vessels anastomose. Two myelinated nerves (MN), numerous exposed nerve endings (NE), a K-cell (K), and oxytalan fibres (arrowed) are also prominent features of this micrograph.

Region: Depth 800 microns, middle third.

Magnification = 6,000x. Bar = 5 microns.

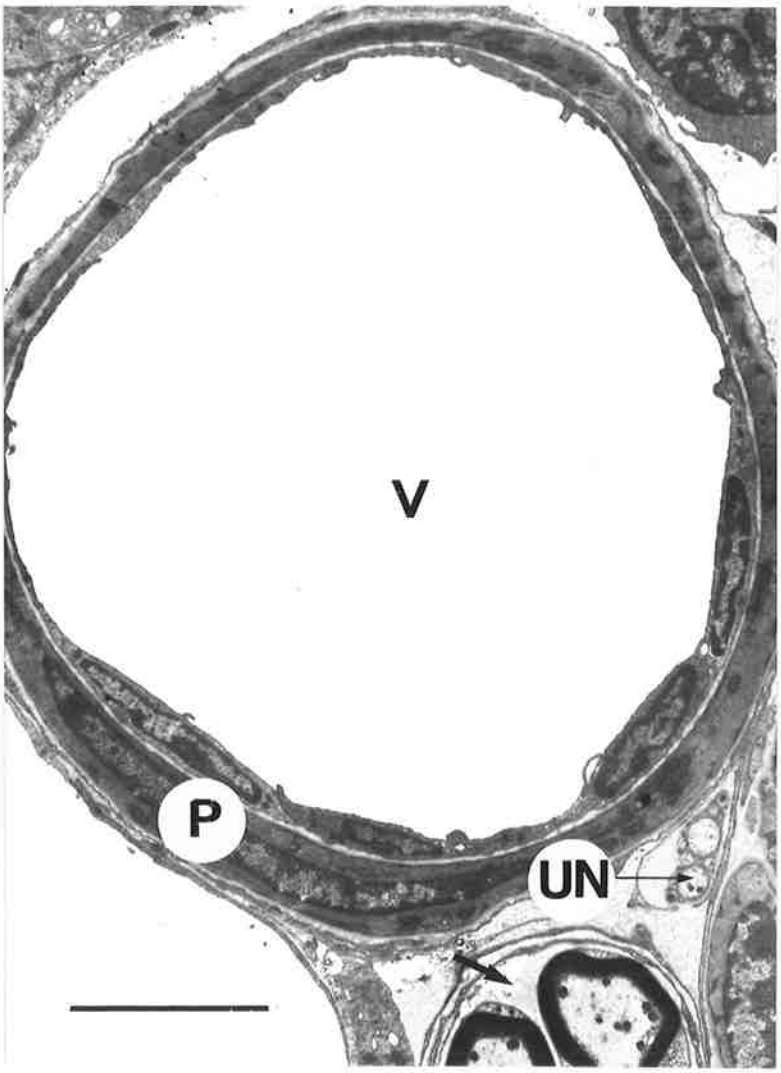


Figure 16: A pericytic postcapillary-sized vessel (V), with a lumenal diameter of 22 microns, completely surrounded by a pericytic or smooth muscle cell investment (P). Such vessels were rarely seen within the ligament. More commonly, in vessels of comparable size pericytic cells were associated with only part of the vessel circumference or were absent. A capsulated myelinated nerve complex (arrowed) and smaller encapsulated unmyelinated nerve axons (UN) are present on this micrograph.

Region: Depth 1000 microns, alveolar third.

Magnification = 5,000x. Bar = 5 microns.

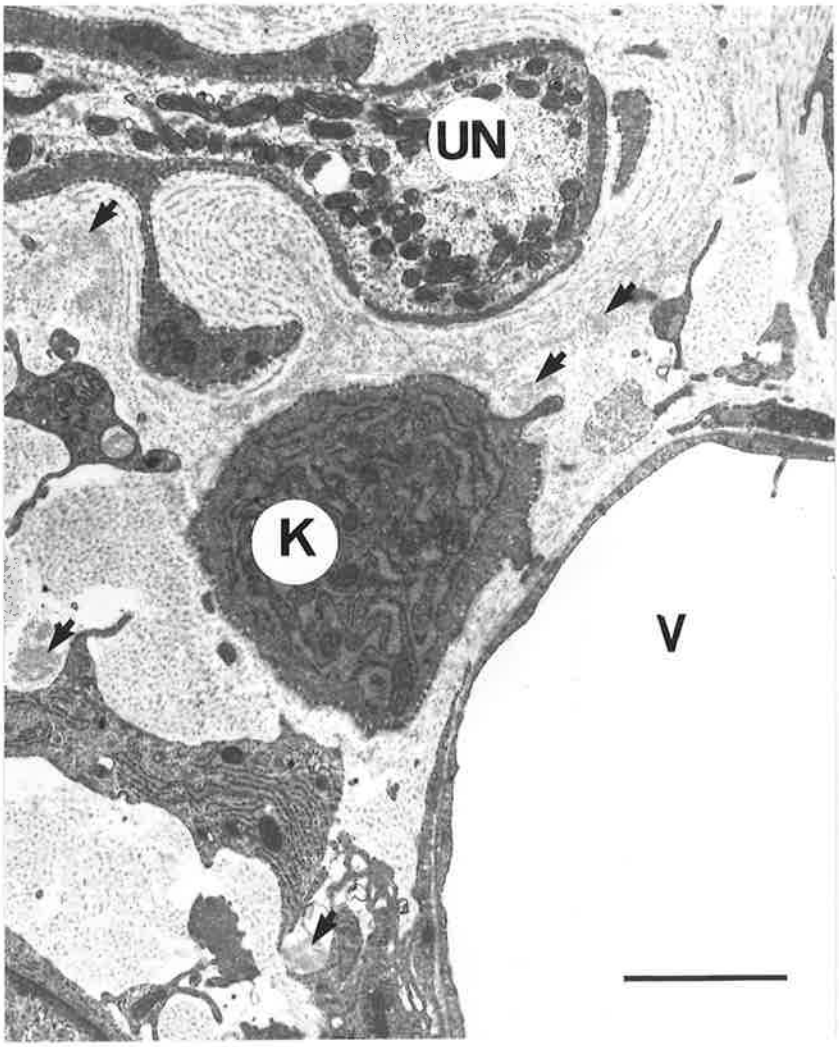


Figure 17: An apericytic postcapillary-sized vessel (V), with a K-cell (K) situated in close proximity to the vessel lumen and a mitochondria rich unmyelinated nerve ending (UN) with a perineural coating containing many microvesicles. Numerous oxytalan fibre bundles (arrowed) are present near these structures. Semi-serial sectioning reveals that the K-cell and unmyelinated nerve communicate (Figure 18).

Region: Depth 400 microns, alveolar third.

Magnification = 7,500x. Bar = 3 microns.

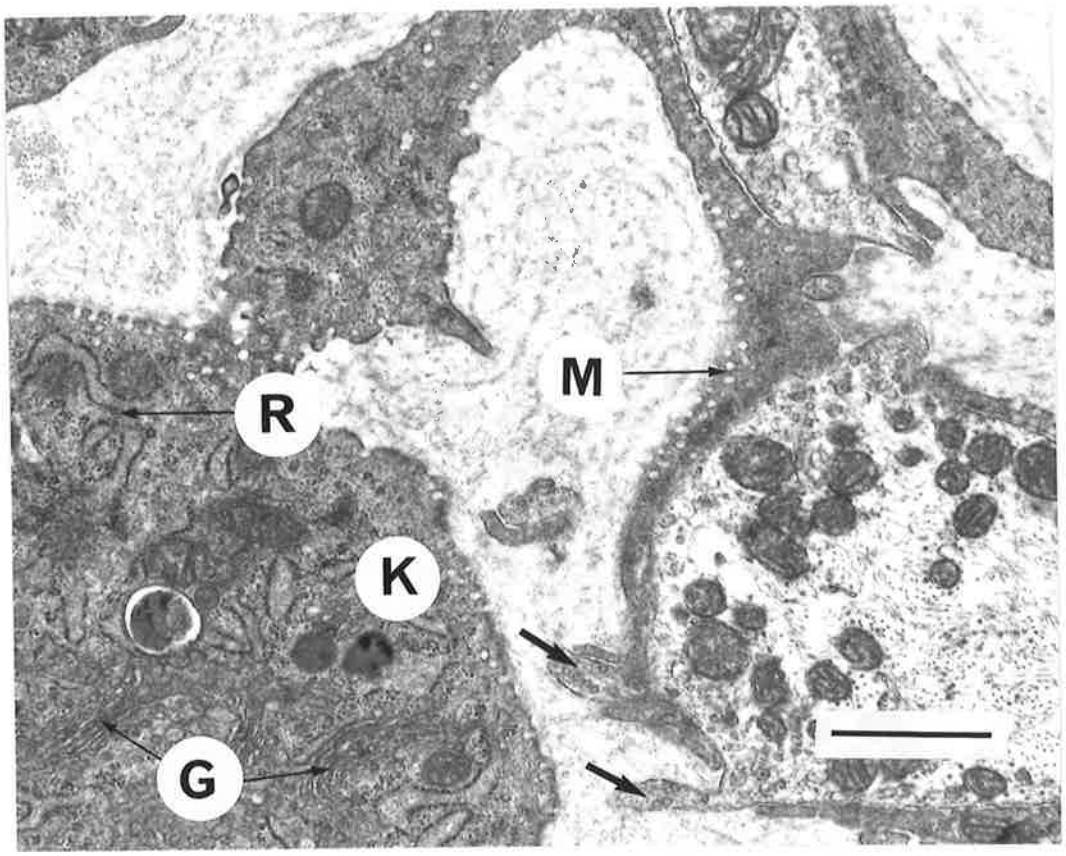


Figure 18: Breaches in the continuity of the perineural investment, with associated extensions of axoplasm containing small granules (arrowed), were present near some K-cells (K). Prominent Golgi apparatus (G) and rough endoplasmic reticulum (R) were present within these K-cells, as were numerous microvesicles (M) in the perineural investment. The function of such structures has not been ascertained.

Region: Depth 850 microns, alveolar third.

Magnification = 14,000x. Bar = 2 microns.

the vessel perimeter of capillary sized vessels. Ultrastructurally, these cells frequently contained many microvesicles, up to 40 nm in diameter, some mitochondria and numerous microfilaments.

The endothelial lining of postcapillary sized vessels varied in thickness from 0.2 microns to 1.7 microns in the region of the cell nucleus. The nucleus of these cells invariably bulged into the connective tissue and, although these cells were sometimes fenestrated, microvesicles were inconspicuous. Microfilaments and mitochondria were present in these cells and up to nine cells formed the vessel perimeter. Lumenal microvillus extensions were present at the endothelial cell junctions of both capillary and postcapillary venule sized vessels.

p. NERVE FIBRES

Myelinated and unmyelinated periodontal nerves were found subjacent to the endothelial lining of blood vessel walls as well as in the parenchyma of the ligament. From the micrographs examined and by semi-serial sectioning, evidence that some unmyelinated axons arose from myelinated nerves that lost their myelinated sheath within the periodontal ligament was found (Figure 19).

Unmyelinated fibres varied in diameter from 0.2 to 2.5 microns and often contained many mitochondria that obscured other cytoplasmic organelles. On occasions, unmyelinated axons lost their Schwann cell coating and were separated from the surrounding collagen fibres by a basement lamina. Myelinated fibres, which were found only in the apical half of the ligament, varied in diameter between 1 and 4 microns, and frequently contained many mitochondria and

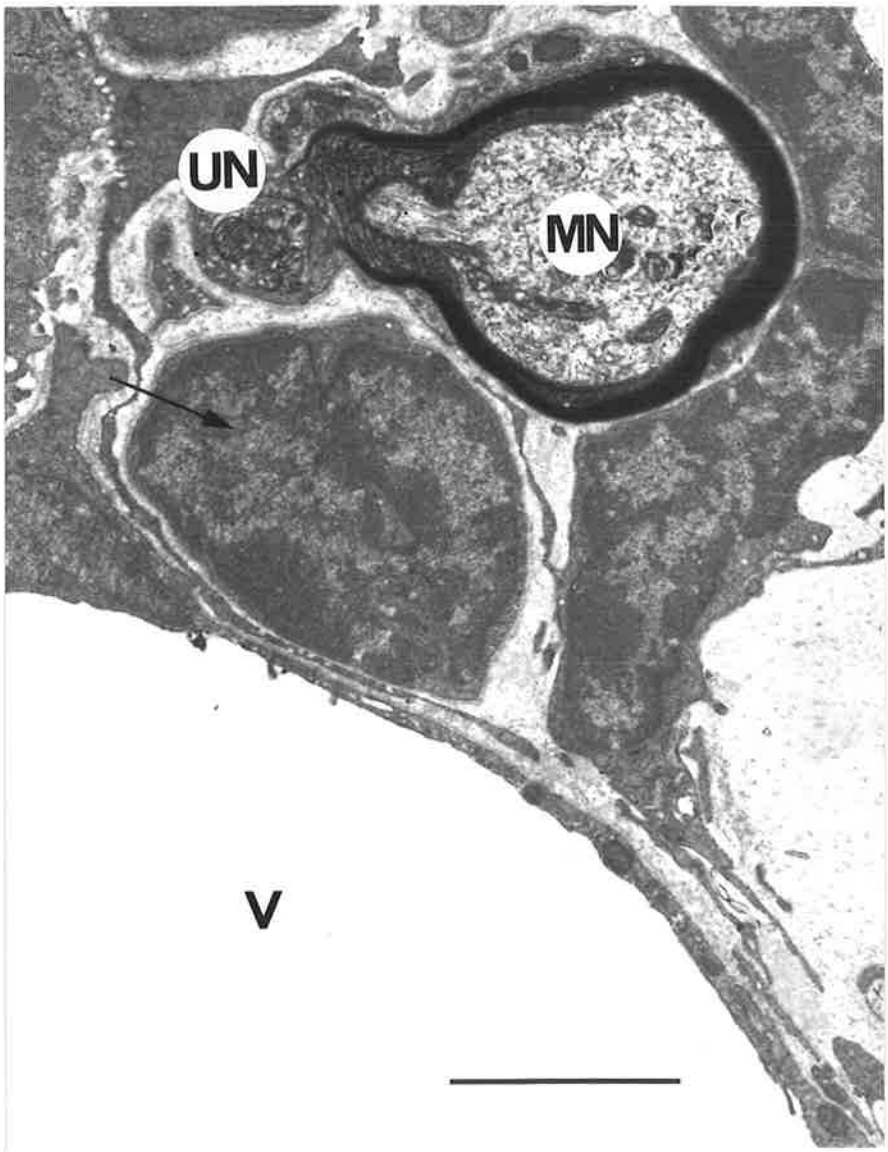


Figure 19: A myelinated periodontal nerve (MN) from which an unmyelinated nerve (UN) is emanating. Interspersed between the unmyelinated nerve and the blood vessel lumen (V) is a cell surrounded by a basement membrane. This cell (arrowed) is presumably either a K-cell or a Schwann cell.

Region: Depth 550 microns, alveolar third.

Magnification = 10,000x. Bar = 3 microns.

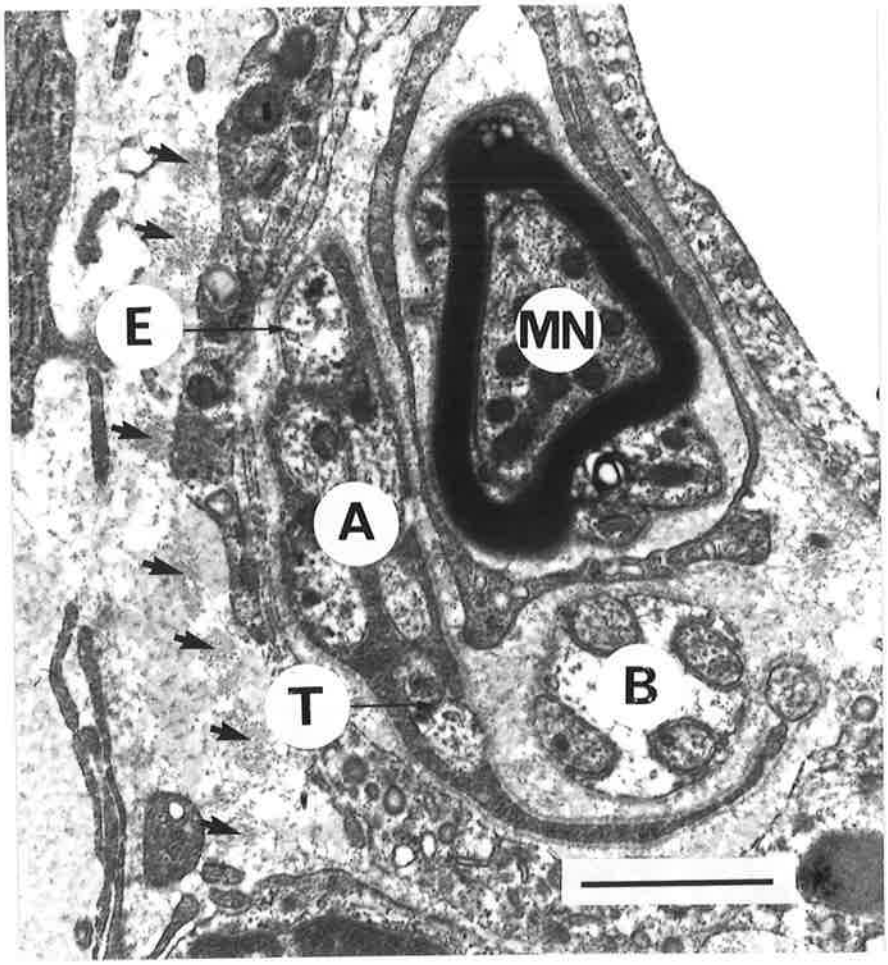


Figure 20: Two distinct arrangements of fine unmyelinated nerve fibres occurred within periodontal blood vessel walls. One group was characterized by an investing cell with darkly staining cytoplasm (A). In this group, the axons were arranged as a flattened structure, with 2 or more axons sometimes enclosed within a single enfolding of Schwann cell cytoplasm (T). Axons in these structures were often partly exposed (E). The other arrangement of axons (B) was as a group of fibres enclosed within a pale staining cell but partially exposed to the surrounding connective tissue. This arrangement frequently assumed a circular form. An encapsulated myelinated nerve axon (MN), 0.3 microns from the lumen of an apericytic blood vessel, is also present as are numerous oxytalan fibre bundles (arrowed).

Region: Depth 600 microns, alveolar third.

Magnification = 22,500x. Bar = 1 micron.

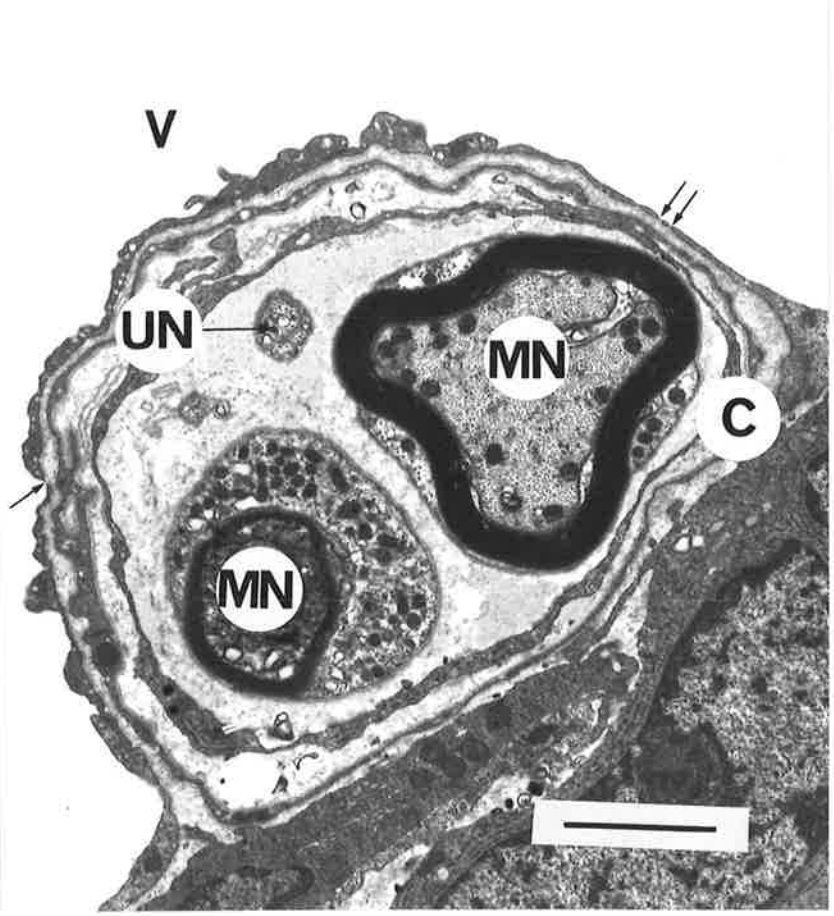


Figure 21: Two myelinated nerves (MN) and two unmyelinated nerves (UN), partially exposed to the surrounding connective tissue and separated by a investing cell with pale staining cytoplasm, form a neural complex that bulges into the vessel lumen (V). Fenestrae (arrowed) in the adjacent endothelial cells were a frequent finding in the vicinity of these structures. The investing capsule (C) of this complex is 2 layers thick in most regions, increasing to 3 layers thick in some areas. The myelinated nerve located towards the lower left of the micrograph has many small mitochondria located in the Schwann cell cytoplasm. An abundance of mitochondria was not seen in Schwann cells at nodes of Ranvier, sectioned longitudinally (Figure 22), but occurred when nerves demyelinated.

Region: Depth 800 microns, middle third.

Magnification = 10,000x. Bar = 2 microns.

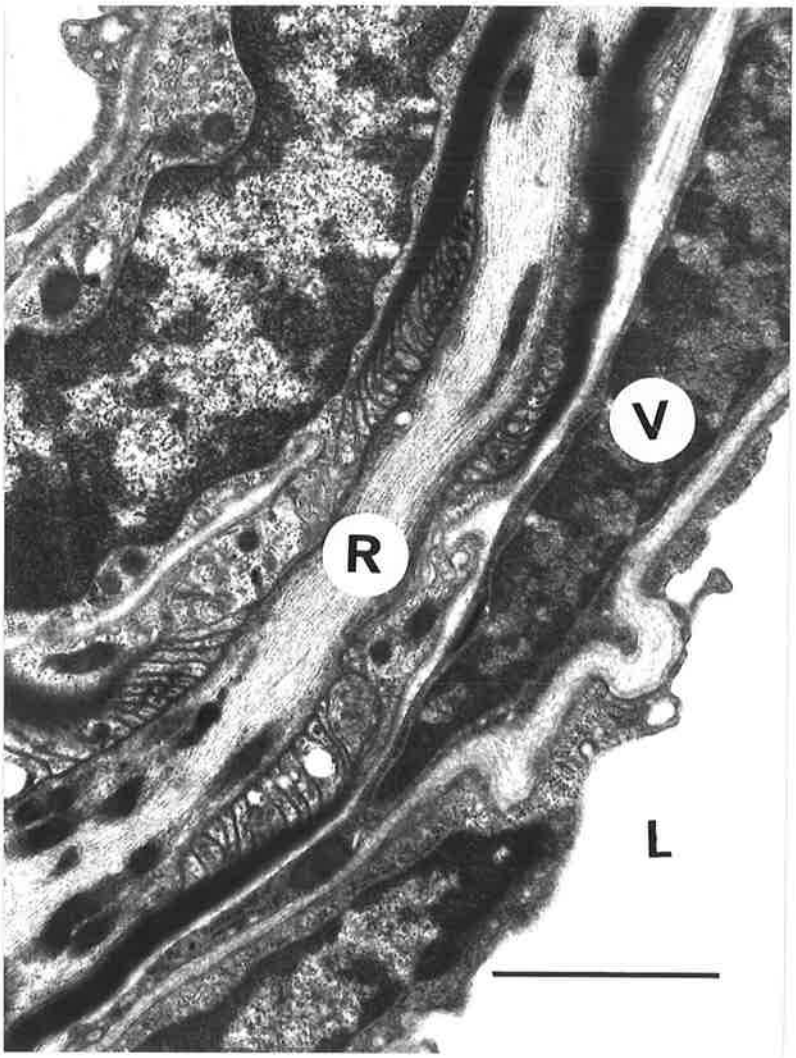


Figure 22: A longitudinal section through a node of Ranvier (R) situated in close proximity to the lumen of an apericytic postcapillary-sized venule (L). A cell without a basement membrane, presumably a veil cell (V), is interspersed between the myelinated nerve and the vessel lumen. Of particular interest is the sparseness of mitochondria situated in the Schwann cell at the node. This is in contrast to the demyelinating nerve in Figure 21.

Region: Depth 800 microns, middle third.

Magnification = 15,000x. Bar = 2 microns.

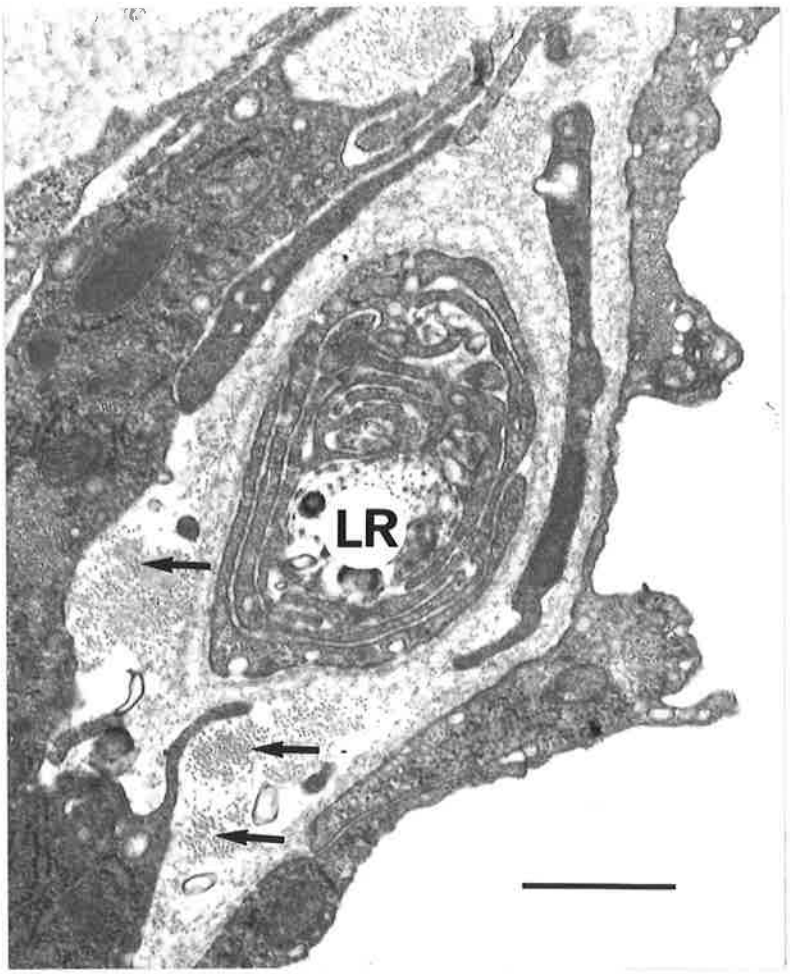


Figure 23: A putative lamellated receptor (LR), with a diameter of 2.5 microns, enclosing a single axon with a diameter of 1.0 micron. This structure was located near the junction of two apericytic postcapillary sized venules. Also present on this micrograph are numerous oxytalan fibre bundles (arrowed).

Region: Depth 450 microns, middle third.

Magnification = 20,000x. Bar = 1 micron.

neurofilaments. Of particular anatomical interest, is the the close apposition of periodontal nerve axons to the endothelial lining of the periodontal blood vessels.

q. NEURAL RECEPTORS

Because of the thickness of sections examined, it is difficult to determine whether a sectioned nerve is a nerve ending, or not. However, semi-serial sectioning demonstrates that free nerve endings occur near periodontal blood vessels and within the principal collagen fibres. On occasions these nerve endings arose from unmyelinated axons that were derived from myelinated nerve fibres.

Two anatomically distinct arrangements of unmyelinated axons occurred in the walls of blood vessels (Figure 20) and it is probable that at least one of these arrangements constitutes a periodontal receptor. Another putative neural receptor occurred as a projection into the blood vessel lumen. These structures consisted of 2 to 3 myelinated nerves with 1 or 2 unmyelinated fibres encapsulated by a cellular sheath 1 to 3 layers thick (Figure 21). The appearance of such extensions into the vessel lumen could be resultant of the plane of section at blood vessel junctions.

Furthermore, a presumptive lamellated periodontal receptor, 2.5 microns in diameter, which was located at the junction of two apericytic postcapillary venules, is represented as Figure 23. K-cells, (section 5.2.i), considered possibly to have a receptor function in rat and mouse incisor ligament, were also found in this mouse molar study.

## 5.3 STATISTICAL ANALYSIS

Initial quantitative assessment of periodontal ligament involved investigation of the pilot data. This data, which was recorded in the categories on the data collection sheet (Appendix 8.14), was collated into the following groups to make the data more manageable and to reduce recording errors.

A - Fibroblasts
Macrophages
Osteoblasts
Cementoblasts
Osteoprogenitor cells
Mesenchymal cells
Veil cells
Neural capsular cells
Cell processes

B - Collagen fibres
Ground substance

D - Nerve fibres
Schwann cells
K-cells

E - Oxytalan fibres

C - Vessel lumen
Endothelial cells
Pericytes
Smooth muscle cells

F - Osteoclasts
Epithelial cells
Degenerated cells
Cementicles
Unknowns

A general linear model, with effects for animal, depth and zone, was used to analyse volumetric proportion and the numbers of different profiles per micrograph. It was found that the volumetric proportions for all the groups analysed were not depth, or animal dependent. Main effects due to animal and side could not be isolated because of the experimental technique. However, variation of all

| | TOOTH THIRD | MIDDLE THIRD | BONE THIRD | TOTAL |
|---|---|---|---|---|
| A. Synthetic Cells | 42.27 ± 1.32 | 35.11 ± 1.40 | 28.74 ± 1.68 | 35.37 ± 0.93 |
| B. Collagen and Ground Substance | 54.49 ± 1.49 | 53.81 ± 1.65 | 44.47 ± 2.3 | 50.90 ± 1.11 |
| C. Vessel Lumen and Perivascular Cells | 0.65 ± 0.33 | 7.66 ± 1.69 | 19.40 ± 2.67 | 9.24 ± 1.19 |
| D. Nerves and Perineural Cells | 0.10 ± 0.06 | 0.91 ± 0.25 | 1.89 ± 0.42 | 0.97 ± 0.17 |
| E. Oxytalan | 0.29 ± 0.07 | 0.79 ± 0.10 | 0.26 ± 0.07 | 0.45 ± 0.05 |
| F. Other | 2.68 ± 0.78 | 0.43 ± 0.14 | 4.21 ± 1.37 | 2.44 ± 0.54 |

TABLE 4: Volumetric proportions of structures (%) (± 1 S.E.M.)

| | TOOTH THIRD | MIDDLE THIRD | BONE THIRD | TOTAL |
|---|---|---|---|---|
| A. Synthetic Cells | 13.83 ± 0.50 | 12.47 ± 0.51 | 7.20 ± 0.38 | 11.17 ± 0.34 |
| B. Collagen and Ground Substance* | 0 | 0 | 0 | 0 |
| C. Vessel Lumen and Perivascular Cells | 0.025 ± 0.02 | 0.47 ± 0.13 | 1.32 ± 0.23 | 0.60 ± 0.10 |
| D. Nerves and Perineural Cells | 0.24 ± 0.11 | 2.15 ± 0.44 | 4.03 ± 0.67 | 2.14 ± 0.29 |
| E. Oxytalan | 6.17 ± 0.58 | 11.47 ± 0.73 | 3.15 ± 0.49 | 6.92 ± 0.43 |
| F. Other | 0.47 ± 0.13 | 0.15 ± 0.05 | 0.39 ± 0.09 | 0.33 ± 0.06 |

TABLE 5: Mean number of profiles per micrograph. (± 1 S.E.M.).

* Collagen fibres and ground substance occur as an interstitial continuum within the ligament, rather than as discrete entities.

groups was statistically significant at the 1% level across the ligament from the tooth to alveolar third.

With the same categorization and statistical routines that were used to analyse the pilot data, results from the final study were investigated for main effects due to animal, side of the mouth, depth, and zone across the ligament. From this analysis it was determined that no effect for animal, side, or depth, was present for any of the categories. However, the variation in both volumetric proportion and the average number of profiles on each micrograph for all categories was again statistically significant, at the 1% level, across the ligament width (Tables 4 and 5).

## 5.4 STATISTICAL CORRELATIONS

A Pearson correlation matrix was constructed for all variables, and the items of particular interest examined in detail. A positive correlation between the number of myelinated and unmyelinated axons in blood vessel walls was statistically significant at the 1% level ($n = 198$, $r = 0.39$). The correlation between the number of myelinated and unmyelinated axons not located in blood vessel walls, was also statistically significant at the 1% level ($n = 198$, $r = 0.25$). However, the correlation between the number of myelinated nerves in blood vessel walls and in the ligament parenchyma was not statistically significant. Similarly, the correlation between unmyelinated axons in blood vessel walls and those in the tissue parenchyma was not statistically significant.

The numbers of oxytalan fibres adjacent to periodontal cells was compared to the number of oxytalan fibres enclosed within collagen

bundles, or located within blood vessel walls. It was found that, whereas the correlation between the distribution of oxytalan fibres adjacent to cells and those enclosed within collagen bundles was statistically significant at the 1% level ($n = 198$, $r = 0.38$), the correlation between oxytalan fibres adjacent to cells and oxytalan fibres within blood vessel walls was not. Similarly, the correlation between the number of oxytalan fibres enclosed in collagen and those located in blood vessel walls, was not statistically significant.

The number of oxytalan fibres adjacent to cells was also correlated with the number of fibroblasts on each micrograph ($n = 198$, $r = 0.46$, $p < 0.01$), but not correlated to any other cell type. Likewise, the number of oxytalan fibres completely enclosed by collagen fibres was found to be correlated to the number of periodontal fibroblasts at the 1% level of significance ($n = 198$, $r = 0.22$). A positive correlation was also found between the number of oxytalan fibres enclosed by collagen and the number of unmyelinated axons not located within blood vessel walls. This correlation was statistically significant at the 5% level ($n = 198$, $r = 0.15$). A statistically significant correlation between the number of oxytalan fibres completely enclosed by collagen bundles and the number of K-cells on each micrograph was also found ($n = 198$, $r = 0.16$, $p < 0.05$).

The correlation between the number of oxytalan fibres in the blood vessel wall and the number of vessels was statistically significant only for apericytic postcapillary-sized venules ($n = 198$, $r = 0.14$, $p < 0.05$), and pericytic capillary-sized vessels ($n = 198$, $r = 0.28$, $p < 0.01$).

| STRUCTURES | | Correlation (r) | Statistical Significance |
|---|---|---|---|
| Myelinated Nerves in Blood Vessel Wall | Unmyelinated Nerves in Blood Vessel Wall | 0.39 | $p<0.01$ |
| Myelinated Nerves in Tissue Parenchyma | Unmyelinated Nerves in Tissue Parenchyma | 0.25 | $p<0.01$ |
| Oxytalan Fibres Adjacent to Cells | Oxytalan Fibres Enclosed in Collagen | 0.38 | $p<0.01$ |
| Oxytalan Fibres Adjacent to Cells | Fibroblasts | 0.46 | $p<0.01$ |
| Oxytalan Fibres Enclosed in Collagen | Fibroblasts | 0.22 | $p<0.01$ |
| Oxytalan Fibres Enclosed in Collagen | Unmyelinated Nerves in Tissue Parenchyma | 0.15 | $p<0.05$ |
| Oxytalan Fibres Enclosed in Collagen | K-Cells | 0.16 | $p<0.05$ |
| Oxytalan Fibres in Blood Vessel Walls | Apericytic Type B Vessels | 0.14 | $p<0.05$ |
| Oxytalan Fibres in Blood Vessel Walls | Pericytic Type A Vessels | 0.28 | $p<0.01$ |
| Myelinated Nerves in Tissue Parenchyma | K-Cells | 0.17 | $p<0.05$ |
| Unmyelinated Nerves in Tissue Parenchyma | K-Cells | 0.20 | $p<0.01$ |
| Apericytic Type B Vessels | K-Cells | 0.19 | $p<0.01$ |

TABLE 6: Statistically significant correlations of interest in the mouse molar periodontal ligament.

K-cells, had a statistically significant positive correlations with:

(i) myelinated nerves not in blood vessel walls, ($n = 198$, $r = 0.17$, $p < 0.05$);

(ii) unmyelinated axons not in blood vessel walls ($n = 198$, $r = 0.20$, $p < 0.01$); and,

(iii) apericytic postcapillary sized venules ($n = 198$, $r = 0.19$, $p < 0.01$).

## 5.5 STEREOLOGICAL ASSESSMENT

Volumetric proportion of individual components was then assessed. These results are presented for the tooth, middle and bone thirds of the ligament, in Tables 7a to 12a. Similarly, the average numbers of profiles per micrograph are presented for the tooth, middle and bone thirds of the ligament, in Tables 7b to 12b.

Comparison of stereological parameters has been limited to different zones across the ligament, because variation in the data from which these parameters were calculated was not statistically significant with animal, side of the mouth, or depth along the tooth surface. The stereological formulae used in this study assume that all nerve axons and oxytalan fibres have been sectioned transversely, and that axons, blood vessels and oxytalan fibres are cylindrical in shape. Calculation of minimum interstructural distance assumes that each structure is either located at the corner of an equilateral triangle ($I.S.D._{.}^{1}$) or at the corner of a square ($I.S.D._{.}^{2}$). Calculation of surface area per unit volume was calculated by assuming that vessels of a given type were monodispersed cylinders with a diameter equivalent to

TABLE 7a SYNTHETIC CELLS AND MACROPHAGES

| PROFILE | COARSE | | | | FINE |
|---|---|---|---|---|---|
| | Tooth Third | Middle Third | Bone Third | Overall | Ligament |
| Fibroblasts | 35.7 | 28.7 | 20.4 | 28.4 | |
| Macrophages | 1.1 | 2.5 | 2.1 | 1.9 | |
| Cell Processes | 3.8 | 3.3 | 3.9 | 3.7 | |
| Veil Cells | 0 | 0.3 | 0.4 | 0.2 | 0.1 |
| Perineural Capsule | 0 | 0.2 | 0.1 | 0.1 | 0.1 |
| Osteoblasts | 0 | 0 | 1.1 | 0.3 | 0.4 |
| Mesenchymal Cells | 0 | 0.1 | 0.3 | 0.1 | 0.1 |
| Osteoprogenitor | 0 | 0 | 0.7 | 0.2 | 0.3 |
| Cementoblasts | 1.6 | 0 | 0 | 0 | 0.5 |

GROUP A: Volume proportion of individual components, (using coarse and fine grids).

TABLE 7b SYNTHETIC CELLS AND MACROPHAGES

| PROFILE | Tooth Third | Middle Third | Bone Third | Overall |
|---|---|---|---|---|
| Fibroblasts | 12.39 | 11.21 | 5.61 | 9.73 |
| Macrophages | 0.61 | 0.97 | 0.70 | 0.76 |
| Cell Processes | 0 | 0 | 0 | 0 |
| Veil Cells | 0.03 | 0.20 | 0.26 | 0.16 |
| Perineural Capsule | 0 | 0.18 | 0.10 | 0.10 |
| Osteoblasts | 0 | 0 | 0.30 | 0.10 |
| Mesenchymal Cells | 0 | 0.05 | 0.09 | 0.05 |
| Osteoprogenitor | 0 | 0 | 0.24 | 0.08 |
| Cementoblasts | 0.75 | 0 | 0 | 0.25 |

GROUP A: Number of profiles per micrograph (using the convention of forbidden lines).

TABLE 8a VESSEL LUMEN AND PERIVASCULAR CELLS

| PROFILE | COARSE | | | | FINE |
|---|---|---|---|---|---|
| | Tooth Third | Middle Third | Bone Third | Overall | Ligament |
| Pericytic Capillaries | 0 | 0 | 0.2 | 0.1 | 0.1 |
| Apericytic Capillaries | 0.1 | 1.2 | 1.0 | 0.8 | 0.8 |
| Pericytic PCV | 0 | 0.8 | 4.0 | 1.6 | 1.6 |
| Nonpericytic PCV | 0 | 4.2 | 10.8 | 5.1 | 4.9 |
| Endothelium | 0.3 | 1.6 | 3.3 | 1.7 | 1.5 |
| Pericytes | 0 | 0.1 | 0.8 | 0.3 | 0.3 |

GROUP B: Volume proportion of individual components, (using coarse and fine grids).

TABLE 8b VESSEL LUMEN AND PERIVASCULAR CELLS

| PROFILE | Tooth Third | Middle Third | Bone Third | Overall |
|---|---|---|---|---|
| Pericytic Capillaries | 0 | 0 | .05 | .02 |
| Apericytic Capillaries | 0.02 | 0.20 | 0.21 | 0.14 |
| Pericytic PCV | 0 | 0.05 | 0.15 | 0.07 |
| Nonpericytic PCV | 0 | 0.08 | 0.42 | 0.16 |
| Endothelium* | 0 | 0 | 0 | 0 |
| Pericytes | 0 | 0.15 | 0.38 | 0.18 |

GROUP B: Number of profiles per micrograph (using the convention of forbidden lines).

* Numbers of profiles not counted.

TABLE 9a COLLAGEN AND GROUND SUBSTANCE

| | COARSE | | | | FINE |
|---|---|---|---|---|---|
| PROFILE | Tooth Third | Middle Third | Bone Third | Overall | Ligament |
| Collagen Fibres | 52.8 | 52.1 | 42.3 | 49.2 | - |
| Ground Substance | 1.9 | 1.9 | 2.3 | 2.1 | |

GROUP C: Volume proportion of individual components, (using coarse and fine grids).

TABLE 9b COLLAGEN AND GROUND SUBSTANCE

| PROFILE | Tooth Third | Middle Third | Bone Third | Overall |
|---|---|---|---|---|
| Collagen Fibres* | 0 | 0 | 0 | 0 |
| Ground Substance* | 0 | 0 | 0 | 0 |

GROUP C: Number of profiles per micrograph (using the convention of forbidden lines).

* Numbers of profiles not counted.

TABLE 10a NERVES AND PERINEURAL CELLS

| PROFILE | COARSE | | | | FINE |
|---|---|---|---|---|---|
| | Tooth Third | Middle Third | Bone Third | Overall | Ligament |
| Unmy. in Vessel Wall | 0* | 0.1 | 0.2 | 0.1 | 0.1 |
| Unmy. in Body Ligament | 0* | 0.3 | 0.2 | 0.2 | 0.2 |
| Myel. in Vessel Wall | 0 | 0 | 0.2 | 0.1 | 0.1 |
| Schwann Cells | 0* | 0.3 | 0.5 | 0.2 | 0.2 |
| K-Cells | 0 | 0.6 | 0.3 | 0.3 | 0.3 |

GROUP D: Volume proportion of individual components, (using coarse and fine grids).

* These profiles occurred too infrequently in this region of the ligament to be detected with the coarse grid.

TABLE 10b NERVES AND PERINEURAL CELLS

| PROFILE | Tooth Third | Middle Third | Bone Third | Overall |
|---|---|---|---|---|
| Unmy. in Vessel Wall | 0.06 | 0.50 | 1.64 | 0.73 |
| Unmy. in Body Ligament | 0.08 | 0.82 | 0.91 | 0.60 |
| Myel. in Vessel Wall | 0 | 0.02 | 0.12 | 0.05 |
| Myel. in Body Ligament | 0 | 0.03 | 0.06 | 0.03 |
| Schwann Cells | 0.10 | 0.48 | 0.91 | 0.50 |
| K-Cells | 0 | 0.12 | 0.29 | 0.14 |

GROUP D: Number of profiles per micrograph (using the convention of forbidden lines).

TABLE 11a OXYTALAN FIBRES

| PROFILE | COARSE | | | | FINE |
|---|---|---|---|---|---|
| | Tooth Third | Middle Third | Bone Third | Overall | Ligament |
| Adjacent to Cells | 0.3 | 0.8 | 0.2 | 0.4 | 0.4 |
| Within Collagen | 0* | 0.3 | 0* | 0.1 | 0.1 |
| In Vessel Wall | 0* | 0* | 0.2 | 0.1 | 0.1 |

GROUP E: Volume proportion of individual components, (using coarse and fine grids).

* These profiles occurred too infrequently in this region of the ligament to be detected with the coarse grid.

TABLE 11b OXYTALAN FIBRES

| PROFILE | Tooth Third | Middle Third | Bone Third | Overall |
|---|---|---|---|---|
| Adjacent to Cells | 5.47 | 8.77 | 1.89 | 5.38 |
| Within Collagen | 0.67 | 1.95 | 0.64 | 1.09 |
| In Vessel Wall | 0.03 | 0.74 | 0.62 | 0.46 |

GROUP E: Number of profiles per micrograph (using the convention of forbidden lines).

TABLE 12a OTHER

| PROFILE | COARSE | | | FINE | |
|---|---|---|---|---|---|
| | Tooth Third | Middle Third | Bone Third | Overall | Ligament |
| Epithelial Cells | 2.1 | 0 | 0 | 0.7 | 0.7 |
| Osteoclasts | 0 | 0 | 1.9 | 0.6 | 0.7 |
| Haemopoetic Cells | 0 | 0 | 0 | 0 | 0 |
| Unknowns | 0.1 | 0.3 | 0.5 | 0.3 | 0.2 |
| Cementicles | 0* | 0 | 0 | 0 | 0 |
| Degenerating Cells | 0.4 | 0.1 | 1.7 | 0.7 | 0.6 |

GROUP F: Volume proportion of individual components, (using coarse and fine grids).

* These profiles occurred too infrequently in this region of the of the ligament to be detected with the coarse grid.

TABLE 12b OTHER

| PROFILE | Tooth Third | Middle Third | Bone Third | Overall |
|---|---|---|---|---|
| Epithelial Cells | 0.30 | 0 | 0 | 0.10 |
| Osteoclasts | 0 | 0 | 0.09 | 0.03 |
| Haemopoetic Cells | 0 | 0 | 0 | 0 |
| Unknowns | 0 | 0.11 | 0.12 | 0.08 |
| Cementicles | 0.09 | 0 | 0 | 0.03 |
| Degenerating Cells | 0.08 | 0.04 | 0.18 | 0.10 |

GROUP F: Number of profiles per micrograph (using the convention of forbidden lines).

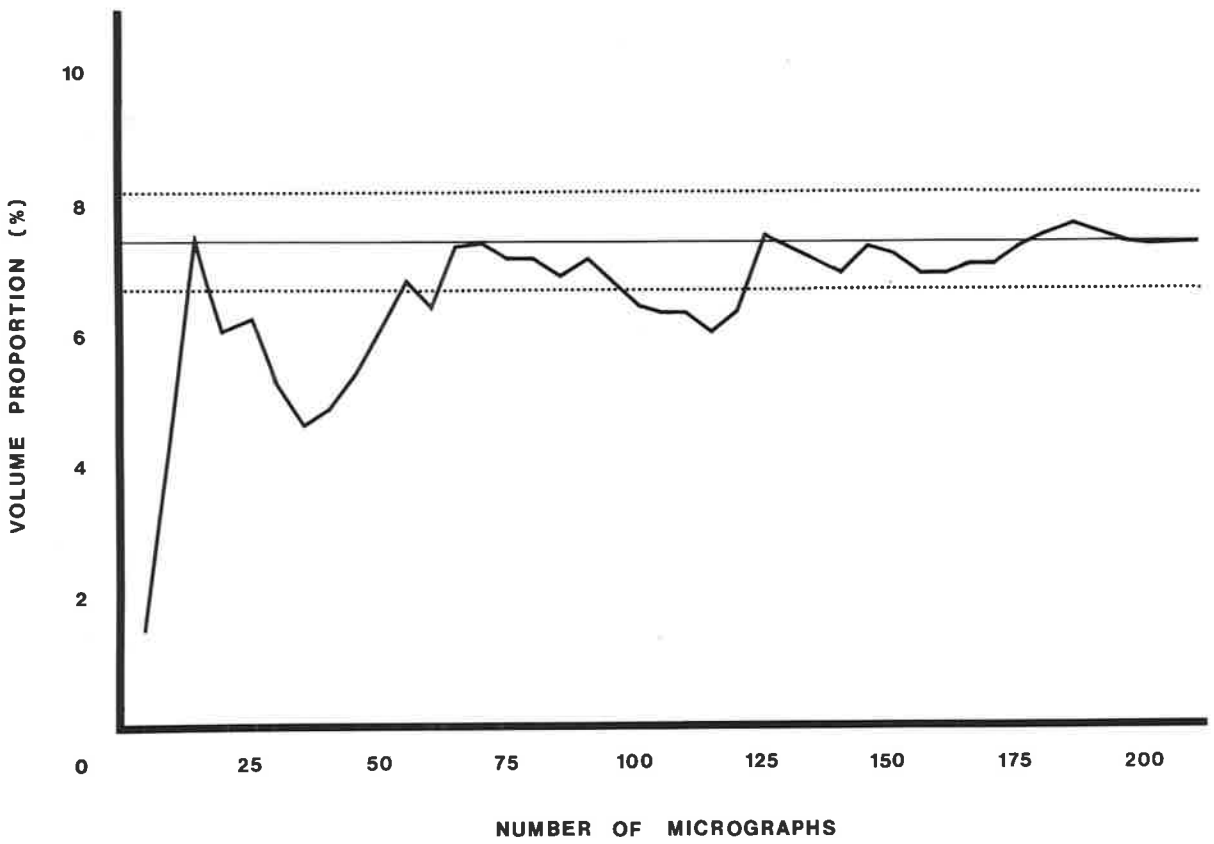


Figure 24: A plot of the progressive mean for periodontal blood vessel volume. This figure demonstrates that the quantification of 120 quadrats is required to achieve a confidence limit of $\pm$ 10%.

that calculated for the region investigated. These assumptions allow direct comparison with other research.

For nerve axons and oxytalan fibres volumetric proportion ($V_v$), the length of structure per unit volume ($L_v$), minimum interstructural distance (I.S.D.[1], I.S.D.[2]), mean cross-sectional area (A) and mean caliper diameter (d), are presented. For blood vessels, the surface area per unit volume ($S_v$), and radius of the cylinder, concentric to the blood vessel, through which nutrients from that blood vessel diffuse, termed Krogh's cylinder (K), have also been included.

To determine the confidence limits with which stereological parameters have been calculated, an investigation of progressive means was undertaken. From the examination of the initial results, it was apparent that blood vessels showed the greatest variation in volumetric and numeric proportion. Figure 24 illustrates that examination of 66 test quadrats, as examined when individual zones across the ligament were investigated, achieves a confidence limit of ±20%. In the analysis of the periodontal ligament as a complete entity, 198 test quadrats were examined, giving a confidence interval of ±5%.

a. CELLS

Of the periodontal cells investigated, fibroblasts, pericytes and epithelial cells were considered of particular interest. Quantitatively, fibroblasts occupied 35.6 ± 0.2% of the tooth third ligament volume. In the middle and bone thirds, fibroblasts had a volumetric proportion of 28.9 ± 0.2% and 20.3 ± 0.2%, respectively (Table 7.a). This is equivalent to 35.7%, 30.9% and 24.1% of the

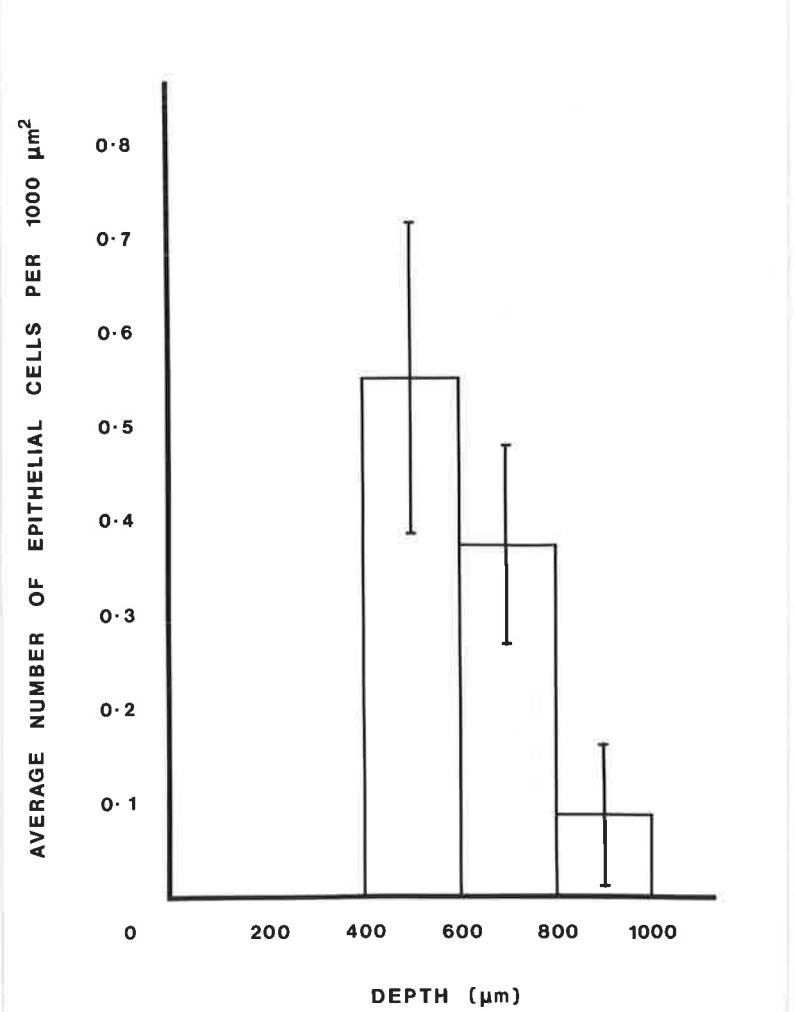


Figure 25: Variation in the mean number of epithelial cells in the tooth third of the ligament.

Bar = ±1 standard error of the mean.

extravascular volume in the tooth, middle and bone thirds of the ligament.

Pericytes were not found in the tooth third of the periodontal ligament and comprised 0.1% and 0.8% in the middle and alveolar lateral thirds of the ligament, respectively (Table 8.a).

Epithelial cells were found exclusively in the tooth third of the ligament. A histogram of the variation in number of these structures per micrograph with depth (Figure 25) indicates that epithelial cells were not found at depths less than 400 microns. These cells were seen most frequently at the mid-root depths of the ligament, there being less epithelial cells towards the tooth apex. This feature has not been examined in detail.

b. OXYTALAN FIBRES

The length of oxytalan fibres within the periodontal ligament averaged 1251.5 x $10^3$cm/cm$^3$. The minimum interfibre distance was calculated to be 9.6 microns. The mean caliper diameter, of approximately 0.7 microns, compares favourably to their true anatomical size.

A statistically significant variation in the number of oxytalan fibres, and their volumetric proportions, occurred across the ligament from the tooth surface to the alveolar bone. Of the total oxytalan fibre length, 55% occurred in the middle third, 30% in the tooth third and 15% in the bone third of the ligament (Table 13).

Furthermore, 78% of the periodontal oxytalan fibres were located adjacent to periodontal cells, 14% were completely enclosed by

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) |
|---|---|---|---|---|---|---|
| Tooth Third | 0.29 ± 0.07 (22%) | 1128.6 (30%) | 0.3 | 0.6 | 10.1 | 9.4 |
| Middle Third | 0.79 ± 0.10 (59%) | 2048.2 (55%) | 0.4 | 0.7 | 7.5 | 7.0 |
| Bone Third | 0.26 ± 0.07 (19%) | 577.6 (15%) | 0.5 | 0.8 | 14.1 | 13.2 |

TABLE 13: Stereological parameters for all oxytalan fibres in different zones within the ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) |
|---|---|---|---|---|---|---|
| Adjacent to cells | 0.35 ± 0.04 (78%) | 976.5 (78%) | 0.4 | 0.7 | 10.9 | 10.1 |
| In collagen bundles | 0.06 ± 0.02 (14%) | 197.1 (16%) | 0.3 | 0.6 | 24.2 | 22.5 |
| In blood vessel walls | 0.04 ± 0.01 (8%) | 84.4 (6%) | 0.5 | 0.8 | 37.0 | 34.4 |

TABLE 14: Stereological parameters for oxytalan fibres, adjacent to cells, in collagen bundles,and in blood vessel walls, within the ligament.

collagen bundles and 8% occurred within the walls of blood vessels (Table 14). However, the proportion of oxytalan fibres adjacent to cells, within collagen bundles and in blood vessel walls was not uniform across the ligament. In the tooth third 89% of the periodontal oxytalan was adjacent to cells, whereas in the middle and bone thirds there was 76% and 60% of the total oxytalan in that third, adjacent to cells, respectively. The increase in oxytalan fibre length in blood vessel walls from the tooth to bone third was proportional to the increase in blood vessel length, such that the number of fibres associated with each vessel remained constant. A gradual increase in the relative proportion of oxytalan enclosed within collagen bundles occurred from 10% in the tooth third of the ligament to 20% in the bone third.

c. BLOOD VESSELS

A number of stereological parameters have been calculated for periodontal blood vessels. These indicate that the total blood vessel length within the ligament was 47.4 x $10^3 cm/cm^3$, the total surface area was 182.4 x $10^3 cm^2/cm^3$ and the average distance between vessels was 40.5 microns. However, because of the distinct vessel types, categorization of the vessels was necessary. To minimize problems of classification arising from the plane of section, and so as not to infer a physiological function in the nomenclature, vessels were categorized as capillary-sized type A, vessels and postcapillary venule-sized type B vessels. Of the type B vessels, those devoid of any pericytic cellular investment, in the part of the vessel examined, comprised 70% of the vessels type. Those in which pericytes were

| | $V_v$ (%) | $L_v$ ($\times 10^3 cm/cm^3$) | Sv ($\times 10^3 cm^2/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) | KROGH'S CYLINDER ($\mu m$) |
|---|---|---|---|---|---|---|---|---|
| Tooth Third | 0.16 ± 0.24 (5%) | 2.8 (2%) | 7.5 (1%) | 58.4 | 13.7 | 204.1 | 189.9 | 107.1 |
| Middle Third | 6.19 ± 1.42 (28%) | 53.4 (38%) | 203.9 (33%) | 115.9 | 12.2 | 46.5 | 43.3 | 24.4 |
| Bone Third | 15.78 ± 2.3 (67%) | 83.2 (60%) | 406.4 (66%) | 189.3 | 15.5 | 37.2 | 34.6 | 19.5 |
| Total Ligament | 7.46 ± 1.0 | 46.5 | 208.8 | 160.3 | 14.3 | 49.8 | 46.3 | 26.1 |

TABLE 15: Stereological parameters for all blood vessels in different lateral thirds within the ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | $S_v$ ($x10^3 cm^2/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) | KROGH'S CYLINDER ($\mu m$) |
|---|---|---|---|---|---|---|---|---|
| Capillary Vessels | 0.92 ± 0.21 | 28.4 | 57.3 | 32.3 | 6.4 | 63.7 | 59.3 | 33.5 |
| Type A | (12%) | (60%) | (31%) | | | | | |
| Venous Vessels | 6.54 ± 0.97 | 19.0 | 125.1 | 343.7 | 20.9 | 77.9 | 72.5 | 40.9 |
| Type B | (88%) | (40%) | (69%) | | | | | |

TABLE 16: Stereological parameters for venular and capillary sized vessels within the ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | $S_v$ ($x10^3 cm^2/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) | KROGH'S CYLINDER ($\mu m$) |
|---|---|---|---|---|---|---|---|---|
| Tooth Third | 0.16 ± 0.12 (6%) | 2.8 (3%) | 7.5 (4%) | 58.4 | 8.6 | 204.1 | 189.9 | 107.1 |
| Middle Third | 1.28 ± 0.45 (46%) | 35.2 (41%) | 75.1 (44%) | 36.3 | 6.8 | 57.3 | 53.3 | 30.1 |
| Bone Third | 1.32 ± 0.41 (48%) | 47.2 (56%) | 88.3 (52%) | 27.9 | 6.0 | 49.5 | 46.0 | 26.0 |

TABLE 17: Stereological parameters for capillary sized Type A vessels within different lateral thirds of the ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | $S_v$ ($x10^3 cm^2/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) | KROGH'S CYLINDER ($\mu m$) |
|---|---|---|---|---|---|---|---|---|
| Tooth Third | 0 (0%) | 0 (0%) | 0 (0%) | | | | | |
| Middle Third | 1.9 ± 1.4 (25%) | 18.3 (34%) | 106.2 (29%) | 269.2 | 18.5 | 79.5 | 74.0 | 41.8 |
| Bone Third | 14.5 ± 2.3 (75%) | 36.1 (66%) | 256.2 (71%) | 400.5 | 22.6 | 56.5 | 52.6 | 29.7 |

TABLE 18: Stereological parameters for Type B vessels within different regions in the ligament. Because of vessel size, some overlap of vessels occurred between adjacent lateral thirds of the ligament.

present, in the region of the vessel examined, constituted the remaining 30%.

When the stereological parameters for all blood vessels within different regions across the ligament were considered, it was apparent that great variation in the periodontal vasculature occurred across the ligament width, and in fact this variation was statistically significant. In the tooth, middle and bone thirds, blood vessels occupied 0.2 ± 0.2%, 6.2 ± 1.4% and 15.8 ± 2.3% of the ligament volume, respectively (Table 15). Changes in vessel length per unit volume paralleled changes in volumetric proportion.

Stereological parameters for type A and type B blood vessels within the ligament demonstrate that these vessels are structurally dissimilar. Type A vessels had a mean diameter of 6.4 microns, whereas type B vessels averaged 20.9 microns in diameter. The type B vessels occupied 6.54 ± 0.97% of the ligament volume, this being 88% of the total periodontal vascular pool. However, because of their size they contributed only 69% to the total periodontal blood vessel surface area and comprised only 40% of the total vessel length. By comparison, the more numerous type A vessels, contained only 12% of the periodontal blood volume, gave rise to 31% of the endothelial surface area and comprised 60% of the total periodontal blood vessel length (Table 16).

Furthermore, a difference in the distribution and volumetric proportion of these two blood vessel types occurred within the different lateral zones of the ligament. Whereas type A vessels constituted 100% of the vessels in the tooth third, they numerically comprised only 67% of the vessels in the middle third and 57% of the

vessels in the bone third of the ligament. Volumetrically, type B vessels, comprised none of the blood volume in the tooth third of the ligament, constituted 79% of the blood volume in the middle third, and 92% of the periodontal blood volume in the bone third of the ligament.

Investigation of the distribution of type A vessels within the ligament (Table 17) indicates that of the total population of these vessels, 3% occurred in the tooth third, 41% in the middle third and 56% in the bone third of the ligament where the type A vessel length was 47.2 x $10^3$cm/cm$^3$. Calculation of minimum intervessel distance indicates that type A vessels are located 189.9 microns apart in the tooth third, 53.3 microns apart in the middle third and 46.0 microns apart in the bone third of the ligament.

For type B vessels 34% of the vessel length occurs in the middle third of the ligament and 66% in the bone third. Volumetrically, 75% of the blood contained within the type B vessels is also contained in the alveolar third of the ligament (Table 18). These figures indicate a disproportionately high vascularity of type B vessels in the bone third of the ligament.

d. NERVES

Stereological parameters for periodontal nerve fibres demonstrate that these structures have a length per unit volume approximately five times that of blood vessels and about one-fifth that of oxytalan fibres. The number of axons varies across the width of the ligament as does the proportion of myelinated and unmyelinated axons and the proportion of axons located in blood vessel walls.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) |
|---|---|---|---|---|---|---|
| Tooth Third | 0.10 ± 0.05 (6%) | 25.0 (3%) | 3.6 | 2.2 | 68.0 | 63.3 |
| Middle Third | 0.46 ± 0.13 (33%) | 243.5 (32%) | 1.9 | 1.5 | 21.8 | 20.2 |
| Bone Third | 0.85 ± 0.20 (61%) | 500.0 (65%) | 1.7 | 1.5 | 15.2 | 14.1 |

TABLE 19: Stereological parameters for all nerve axons in different lateral thirds of the ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) |
|---|---|---|---|---|---|---|
| Blood vessel wall | 0.20 ± 0.05 (43%) | 141.2 (55%) | 1.4 | 1.3 | 28.6 | 26.6 |
| Tissue parenchyma | 0.26 ± 0.06 (57%) | 114.7 (45%) | 2.3 | 1.7 | 31.7 | 29.5 |

TABLE 20: Stereological parameters for all axons within blood vessel walls and axons in the parenchyma of the ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) |
|---|---|---|---|---|---|---|
| Myelinated Nerves | 0.12 ± 0.05 (26%) | 13.7 (5%) | 8.7 | 3.3 | 91.2 | 85.2 |
| Unmyelinated Nerves | 0.35 ± 0.06 (74%) | 242.1 (95%) | 1.4 | 1.4 | 21.8 | 20.3 |

TABLE 21: Stereological parameters for myelinated and unmyelinated axons within the ligament.

Comparison of stereological parameters for nerve fibres in different lateral thirds of the ligament (Table 19), demonstrates that in the alveolar third, where 65% of the nerve fibres were located, the minimum distance between axons was 15.2 microns. In the tooth third, where less than 3% of the axons were located, the minimum interstructural distance was 68 microns.

Of the periodontal innervation, 55% of the axon length occurred in blood vessel walls. The remainder of axons were present in the tissue parenchyma, usually enclosed in collagen fibres. Of the fibres present in the tissue parenchyma, a large proportion were orientated parallel to the blood vessels but at a distance to the vessel wall. Nerves located in blood vessel walls were, on average, 0.4 microns smaller than those located elsewhere in the ligament (Table 20).

Myelinated nerve fibres had a mean caliper diameter of 3.3 microns and numerically constituted 5% of the periodontal nerve axons. However, because of their size, these nerve fibres comprised 26% of the periodontal neural volume (Table 21). In the present investigation, myelinated fibres were not present either in the tooth third of the ligament or at depths of less than 400 microns. Of the population of myelinated nerves 20% occurred in the middle third and 80% in the bone third of the ligament. Sixty per cent were present within blood vessel walls and 40% were located elsewhere in the tissue, usually within principal collagen bundles.

By comparison, unmyelinated fibres, which constituted 95% of the periodontal nerve fibre length, were present at all depths along the root surface and were found in the tooth, middle and bone thirds of

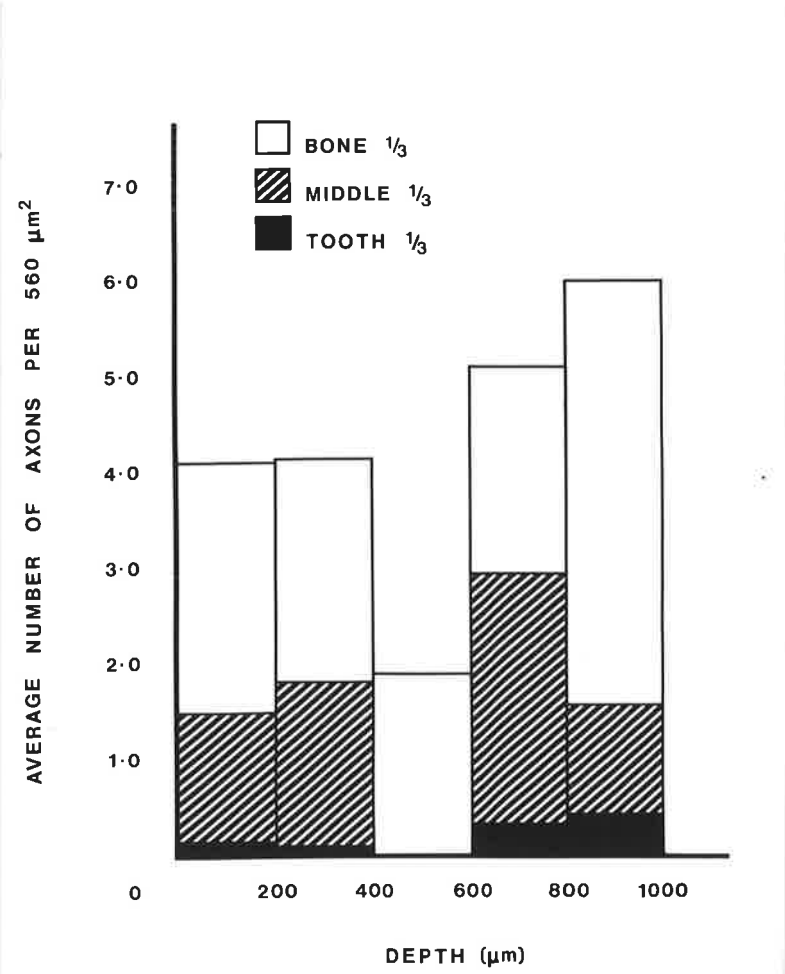


Figure 26: Variation in the mean number of nerve axons per unit area in the tooth, middle and bone thirds of the ligament.

the ligament. These fibres had a mean caliper diameter of 1.4 microns. Quantitatively, 3% of the unmyelinated axons were present in the tooth third of the ligament, and 33% and 64% occurred in the middle and bone thirds respectively. Of the population of unmyelinated axons, 55% occurred in blood vessel walls and 45% in the tissue parenchyma.

A statistically significant variation in the number of nerves and perineural cells with depth was not detected. This was possibly because variation with depth was not uniform across the ligament (Figure 26). However, the most cervical region contained twice as many nerve fibres as the middle of the ligament, and the apical fifth region had an innervation three times that of the middle fifth.

# CHAPTER 6

# DISCUSSION

## 6.1 TECHNICAL AND STEREOLOGICAL CONSIDERATIONS

Morphometry and stereology are closely related but are not synonymous. Whereas morphometry implies the use of quantitative data in the description of structural features, stereology is the three dimensional extrapolation of structures from two dimensional sections on the basis of geometrico-statistical reasoning (Weibel, 1979; Elias, Henning and Schwartz, 1971). Williams (1977) reported that parameters open to study by stereological methods include:

(i) fractional component volumes and surface areas;

(ii) particle numbers and sizes; and,

(iii) mean particle volumes and surface areas.

Errors arising from such studies have been categorized by Underwood (1970) as those arising from:

(i) the experimental limitations, including section distortion, microscope resolution and incorrect point counting;

(ii) improper sampling techniques; and,

(iii) the investigation of non-representative samples.

Of these errors, those arising from the investigation of non-representative tissue samples were considered to be of the greatest magnitude. Although evaluation of all errors arising from the present study is not possible, a detailed discussion of the methodology has been included to indicate why this experimental technique was chosen.

a. ANAESTHESIA

Johnson, Fowler and Zanelli (1976), compared the physiological effects of Nembutal and Urethane. They concluded that reduction in core temperature and blood pressure were more transient and of a lesser magnitude when Urethane was used for anaesthesia. Furthermore, Altura and Weinberg (1979) reported that although Urethane was known to lower blood pressure and vascular responsiveness to noradrenaline, it was still the anaesthetic of choice. More recently, Severs, Keil, Klase and Deen (1981) considered that the stability of blood pressure and maintenance of respiration contributed to the wide spread use of Urethane as an anaesthetic.

Because few pericytes occur in the periodontal ligament it was considered unlikely that the anaesthetic would have any significant direct effect on the periodontal vascular volume.

b. ANTICOAGULANT

Heparinization immediately following anaesthesia was used to circumvent the coagulation of blood elements which might prevent adequate vascular wash out with fixative solution. Although it is possible that heparin may be vasoactive, reports documenting any such effect were not found. Thus, any vasoactive effect of heparin has been neglected.

c. PRIMARY FIXATION

Meek (1976), documented the complementary effects of glutaraldehyde and osmium tetroxide (Table 22). He further stated that

| Osmium tetroxide | Glutaraldehyde |
|---|---|
| Provides excellent electron contrast. | Provides poor electron contrast. |
| Has slow diffusion rate. | Has a rapid diffusion rate. |
| Preserves nucleic acid poorly. | Preserves nucleic acids well. |
| Preserves nucleoproteins well. | Preserves nucleoproteins extremely well. |
| Preserves microtubules poorly if at all. | Preserves microtubules well. |
| Preserves carbohydrates poorly. | Preserves carbohydrates well. |
| Stabilizes unsaturated lipids during fixation. | Does not stabilize lipids during fixation. |
| Preserves phospholipoprotein cell membranes well. | |

TABLE 22: The complementary effects of glutaraldehyde and osmium tetroxide fixatives (from Meek, 1976).

osmium tetroxide had advantages of neither shrinking, swelling nor embrittling tissue, thus enabling ultrathin sections to be cut.

Osmium tetroxide and glutaraldehyde fixatives were first used sequentially for primary fixation by Sabantini, Bensch and Barrnett (1963). In 1966, Trump and Bulger demonstrated that most of the deleterious effects of using these fixatives sequentially, such as lipid extraction and cell shrinkage, could be minimized or avoided if administered simultaneously. Franke, Krien and Brown (1969) considered that the advantages of using these fixatives simultaneously included:

(i) increased contrast distinctiveness of membranes and other lipid containing structures;

(ii) preservation of nucleoprotein structures such as ribosomes and chromatin strands;

(iii) preservation of cytoplasmic microtubules;

(iv) high staining of polysaccharide containing structures; and,

(v) elimination of precipitation contaminants caused by fixation.

Furthermore, Tzeng, Fukuyama and Epstein (1981) stated that the preservation of tonofilaments in rat epidermis was enhanced if simultaneous glutaraldehyde and osmium tetroxide fixation techniques were used. As tonofilaments are present within periodontal epithelial cells (Lester, 1969), this procedure was considered desirable.

Luft and Wood (1963) reported that using osmium tetroxide with cacodylate buffer resulted in the loss of 8% of the original protein during fixation and 4% during dehydration. This rapid release of soluble protein was confirmed by Amsterdam and Schramm in 1966.

The retention of some osmotic activity by cell membranes following fixation with glutaraldehyde has been reported by Jard, Bourguet, Carasso and Favard (1966), Bone and Denton (1971), and Wangensteen, Bachofen and Weibel (1981). Consequently, fixative osmolarity is important when glutaraldehyde fixatives are used. However, Tormey (1965), Glauert (1978), and Wangensteen, Bachofen and Weibel (1981) have determined that primary fixation with osmium tetroxide causes cell membranes to become freely permeable and to lose their osmotic properties.

Bone and Ryan (1972) used a variety of solutions containing both osmium tetroxide and glutaraldehyde to investigate the effect of fixative osmolarity on crab axons. They concluded that fixative osmolarity was not important for fixative solutions containing osmium tetroxide with glutaraldehyde. Because solutions isotonic with, or slightly hypertonic to, periodontal tissue are less damaging to structural components (Lindskog and Blomlöf, 1982; Casley-Smith, 1984), slightly hypertonic solutions were used in the present study.

To avoid osmometer contamination with osmium tetroxide, fixative osmolarity was calculated from graphs published by Maser, Powell and Philpott (1967), who claim an accuracy within 3% of results obtained from freezing point depression. Using these graphs the total osmolarity of the fixative solution was calculated to be 730mosmol. However, Barnard (1976), and Mathieu, Claassen and Weibel (1978) reported the effective osmolarity of glutaraldehyde is less than that of buffer and have documented correction formulae to calculate effective fixative osmolarity. If the correction suggested by Barnard (1976) is used, then the effective osmolarity of the perfusate is

calculated to be 425mosmol. This is slightly hypertonic to mammalian tissues (Weibel, 1979). Williams (1977) suggested the use of slightly hypertonic fixative solutions in which not more than 60% of the total osmolarity was caused by buffer solution. These criteria were fulfilled with the fixative solution used in the present study.

Dextran was included in the perfusate because, being colloidally active, it prevents the artificial enlargement of extravascular tissue spaces by helping to balance the hydrostatic pressure in blood vessels (Casley-Smith, 1984). Hayat (1981) reported that dextran also improves the preservation of the myelin sheath when used with glutaraldehyde fixatives. The pH of the fixative solution was adjusted to pH 7.4, using 1N HCl, as this was determined by Millonig (1962) to be the pH of animal tissues.

Glauert (1978) stated that sucrose and glucose should not be included in osmium tetroxide fixatives because they inhibit proper cross-linking of proteins. Hence, these compounds were not included in the perfusate solution, despite earlier findings by Bahr, Bloom and Friberg (1957), who advocated the use of sucrose to decrease tissue distortion during fixation with osmium tetroxide.

As a buffer system, 0.06M sodium cacodylate was chosen in preference to phosphate buffer because, although unphysiological in action, it:

(i) allows improved tissue stainability following fixation (Wood and Luft, 1965);

(ii) is more resistant to bacterial contamination (Hayat, 1981); and,

(iii) improves the stability and fixation effects of glutaraldehyde with osmium tetroxide (Takahashi, 1980).

d. PERFUSION FIXATION

Glauert (1978) and Hayat (1981) have stated osmium tetroxide will preserve tissue rapidly and uniformly to a depth of 0.25mm, whilst glutaraldehyde will preserve tissue to a depth 0.5mm. Consequently, because of its location, immersion fixation is inappropriate to preserve periodontal ligament. Veerman (1974) reported that perfusion fixation provided uniform fixation of tissue suitable for electron microscopy, this technique first being used to preserve periodontal tissue by Bernick in 1962.

Gil and Weibel (1969) found that whereas a steady perfusion rate caused rapid and substantial tissue oedema, this did not occur when pulsatile perfusion pressures were used. Diastolic and systolic perfusion pressures of 80 to 110mmHg were selected to ensure adequate perfusion with minimal tissue disruption on the basis of publications by Lamport and Baez (1962), Nicoll (1969) and Glauert (1978). A flow rate of 2.5ml/minute was chosen, as this approximated the cardiac output of the mouse (Gurling, 1982).

Thorball and Tranum-Jensen (1983), who investigated vascular reactions to perfusion fixation, concluded that provided hydrodynamic parameters were strictly controlled, preparations could be reliably fixed using perfusion techniques. They also determined that vascular filtration decreased during fixation and recommended that perfusion for 5 to 10 minutes was required to completely saturate tissue.

Consequently, all animals used in this study were perfused for a minimum of five minutes.

Beertsen and Everts (1977), using identical materials and methodology, have demonstrated excellent preservation of periodontal tissue.

e. DIMENSIONAL CHANGES DURING TISSUE PROCESSING

Fejerskov (1971) studied the effects of different dimineralizing media on oral epithelium. Of the agents examined, EDTA allowed the best preservation of cytological detail, and demineralizing at 4°C reduced the amount of cellular dissociation. It was concluded that EDTA demineralization at 4°C was the best procedure to use, and it was on this basis that the demineralizing regime used in the present study, was chosen.

Cell shrinkage during tissue dehydration has been reported by Bahr, Bloom and Friberg (1957) and Kushida (1962). Weibel and Knight (1964), who demonstrated cellular shrinkage following dehydration in alcohol schedules starting with 50% ethanol, reported that dimensional changes remained constant when dehydration was started at 70% ethanol, as it was in the present study.

Kushida (1962) found that cellular shrinkage of 0.6% occurred during polymerization of Epon 812. Weibel and Knight (1964) considered that errors arising from block shrinkage were of little importance when epoxy embedding materials were used. Williams (1977) added that tissues should be embedded in epoxy or polyester resins because of their dimensional stability in the electron beam.

Although tissue staining does not affect tissue dimensions, Williams (1977) advocated the use of heavy metal stains to minimize underestimation of tissue components as a result of poor section contrast. Thus, following embedding in Epon 812 and sectioning, lead citrate and uranyl acetate stains were used in the present study.

Reports of dimensional changes in the periodontal ligament using similar fixation and tissue processing techniques to this project are not available. Because dimensional changes during fixation and tissue preparation are tissue and methodology dependent (Bahr, Bloom and Friberg, 1957; Kushida, 1962), results from other studies cannot be extrapolated directly. However, from consideration of the work by Kushida (1962), and Weibel and Knight (1964), it was concluded that dimensional changes in this study were likely to be less than 1% linearly and less than 2% volumetrically. As dimensional changes during tissue processing were considered minimal and do not effect estimation of volume proportion (Weibel, 1979), they were neglected.

## f. TISSUE SAMPLING

Weibel (1979) stated that stereological calculations are derived from the probability with which section profiles coincide with an appropriate bias-free test system, and that the measurement of stereological parameters assumes random orientation of profiles measured to the plane of section. This demands a rigorous random sampling procedure at all stages of the investigation. However, the sample selected should be representative of the material from which it was obtained. Consequently, micrographs should be well dispersed throughout the tissue. Ebbeson and Tang (1967) demonstrated that when

an inherent periodicity, which could interfere with the sampling regime, is not present, stratified or systematic sampling gives rise to the smallest standard errors. The technique of systematic sampling with a random start, recommended by Weibel (1979), was adopted for the present study.

This sampling technique also allowed the juxtaposition of tissue elements within the ligament to be determined. It was apparent from the pilot study that the number of profiles per unit area and their volumetric proportions were not depth dependent. Furthermore, a structural periodicity within the ligament was not evident in the preliminary results. Thus, it was considered that sampling the tissue at 200 micron intervals would not affect the accuracy of the final data. Blood vessels, nerves and oxytalan fibres were not randomly aligned to the plane of section, and mathematical correction of stereological parameters for these structures was required (Casley-Smith, 1984). The corrections used are accurate to within 3.4% of values derived from randomly orientated sectioning planes, when perfusion techniques are used, and the orientation of the tissue is known (Hoppeler and Mathieu, 1984).

The importance of section thickness has been discussed by Weibel (1979) who reported that:

(i) if the section thickness approaches one twelfth of the profile diameter, then correction for the Holmes effect should be considered;

(ii) if the section thickness is too great then some structures become optically lost because, obscured by other profiles

within the section, they have insufficient contrast to be detected; and,

(iii) the compression distortion of ultrathin sections during cutting, which is inversely proportional to section thickness, leads to an overestimation of those parameters which are not dimensionless.

The size of the profiles, compared to the section thickness, indicated that errors arising from the Holmes effect and lost caps were insignificant for the features examined. Compression distortion was kept to a minimum by:

(i) cutting sections in the silver interference range;

(ii) using a new diamond knife for sectioning; and,

(iii) expanding sections with chloroform vapour before collection.

The use of silver sections, approximately 70 nm thick, had the additional benefit of maximizing profile contrast.

## g. QUADRAT SELECTION

Cruz-Orive and Weibel (1981), who studied sampling designs for stereology, stated that when a section is too large to be measured completely at the required magnification, investigation of different regions on each section should be undertaken. A number of methods are available to select such areas, termed quadrats, for the purposes of stereological study (Weibel, 1979). For this investigation a method of unaligned, fixed orientation, uniform systematic quadrat subsampling was used (Williams, 1977; Weibel, 1979). On the assumption that the grid bars are isotropic, uniform and random with respect to the supported tissue, a uniform random quadrat, representing the tooth

third of the ligament, was selected parallel to the tooth surface adjacent to the intersection of grid bars. Remaining quadrats, representing the middle and alveolar thirds of the ligament, were determined by the position of the first quadrat according to the following criteria:

(i) each quadrat was positioned adjacent to a grid bar;

(ii) each quadrat was completely enclosed by the region which it represented;

(iii) each quadrat contained only periodontal ligament; and,

(iv) all features included within the subsample of tissue studied were free from distortion and processing artifact.

This method of quadrat subsampling was adopted, because unaligned sampling design reduces the possibility of any tissue periodicities coinciding with the quadrats examined (Cruz-Orive and Weibel, 1981).

A TEM micrograph magnification of 3,000x was sufficient for the recognition of cell and blood vessel types and allowed the width of the mouse molar periodontal ligament to be trisected with adjacent micrographs. All photomicrographs were printed at a final magnification of 8,500x. This magnification being standardized using a replicating graticule exposed at the time of tissue examination. Although the micrographic image can become distorted by paper shrinkage during processing (Weibel, 1979), possible errors that could arise from such shrinkage were not measured because they do not affect estimation of the volumetric proportion or the number of cells per micrograph (Casley-Smith, 1984).

The effect of microscope resolution on the direct estimation of surface area per unit volume was discussed by Weibel (1979). Because this parameter was measured indirectly, in accordance with Atherton, Cabric and James (1984), this relationship was neglected.

h. POINT COUNTING

Of all the methods available for estimating volume proportion, Weibel (1979) concluded that systematic point counting was the best. Henning (1967, cited in Williams 1977), suggested that although a random array of points is suitable for anisotropic tissues, it often yields larger statistical errors than a regular array. Because direct analysis of surface density was not undertaken, square lattice test grids were selected in preference to a triangular lattice, or a multipurpose or curvilinear test system.

Test lattice point density was discussed by Weibel (1979), who concluded that test points should be spaced such that only one point lay on the same profile. Following quantification of only ten sections with various density grids, Mathieu, Hoppeler and Weibel (1980) stated, that in order to attain the accuracy of a manual optical picture analyser, 64 points should be placed on each test quadrat. To achieve the best compromise a 35 point grid, with an area value of one point equal to 16 square microns, was placed on each photomicrograph. In accordance with Weibel (1979), it was considered that this test grid would not adequately record rare or small profiles such as oxytalan fibres and nerve axons and a 140 point grid, with an area value of 4 square microns, was subsequently applied to each photomicrograph to

record the volumetric proportion of blood vessels, nerve axons, oxytalan fibres and cells other than fibroblasts.

To determine the number of profiles per unit area the convention of the forbidden lines was used (Figure 27). However, according to Casley-Smith (1984), the size and non-random distribution of periodontal blood vessels makes the convention of forbidden lines inaccurate for determining the number of vessels per unit area. A more appropriate system was used of ascribing the value of one quarter for each side of the photomicrograph that intersected with a blood vessel.

In the calculation of minimum interstructural distance it was assumed that profiles were situated either at the corners of an equilateral triangle or square lattice. Although such a model does not reflect the anatomical situation, it does allow direct comparison with studies of other tissues.

## i. STATISTICAL ACCURACY

Williams (1977) documented that when variation in volumetric proportion and profile number occurs between micrographs, an investigation of progressive means is preferable to the calculation of relative standard error, to determine the minimal sample size required to achieve given confidence limits. Investigation of the standard deviations of blood vessel parameters indicated that variation in the distribution of these structures was greater than that of other structures within the ligament. It was determined, from plotting the cumulative mean, (Figure 24) that quantification of 198 quadrats achieved a confidence interval of ±5%. This represents 34,650 lattice points and 3.64mm$^3$ of tissue.

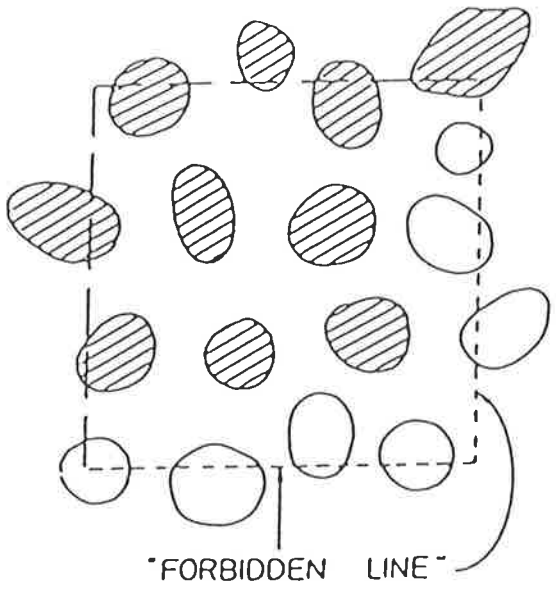


Figure 27. Diagramatic representation of the convention of forbidden lines (Weibel, 1979) with a modification suggested by Casley-Smith (1984). This modification is that profiles that intersect both the "accept" and "forbidden" lines are accepted only when the intersection is greater along the "accept" than the "forbidden" line. If the intersection is greater along the "forbidden" line, then the profile is not counted. Consequently, the shaded profile in the upper right corner is counted but the one in the lower left corner is not.

## 6.2 ULTRASTRUCTURAL FEATURES

Because this study was primarily a stereological assessment of the ligament mesial to the mouse molar, many of the morphological features of the ligament, discussed in the literature review, have not been followed up. A more definitive morphological and morphometric investigation of these features is required to describe and quantify the ultrastructure of periodontal structures within the ligament. However, some features of interest have been included.

### a. FIBROBLASTS

The orientation of periodontal fibroblasts seen in the present study was in accord with earlier findings by Melcher (1976) and Berkovitz and Shore (1982). Although fibroblasts were found to be abundantly occurring pleomorphic cells, categorization of these cells into morphologically distinct types, as Roberts and Chamberlain (1978) have done scanning electron microscopically, was not possible because the effect of plane of section could not be differentiated from actual cell shape.

Ultrastructurally, nuclear morphology was consistent with that documented by Melcher (1976). Findings by Beertsen, Everts and van den Hooff (1974a) that nuclear morphology varies with fibroblast position and cellular activity was not supported by observations in the mouse molar ligament. The abundance and arrangement of rough endoplasmic reticulum, ribosomes and polyribosomes within these cells in the present investigation was similar to that noted by Beertsen, Everts and van den Hooff (1974a), Beertsen and Everts (1977) and Garant and Cho (1979a). The perinuclear location of the Golgi apparatus, described by Garant and Cho (1979a), and mitochondrial morphology described by

Beertsen, Everts and van den Hooff (1974a), Melcher (1976) and Berkovitz and Shore (1982), were confirmed by the present study. However, variations in mitochondrial profile, described by these researchers, were regarded to be resultant from the plane of section rather than separate structural entities.

Microtubules and microfilaments reported by Beertsen, Everts and van den Hooff (1974a) and Garant and Cho (1979a), were not prominent features of periodontal fibroblasts in the present study and the distribution of microtubules was found to be less regular than that described previously by Beertsen, Everts and van den Hooff (1974a). Cilia, found in the present investigation were ultrastructurally similar to those described by Beertsen, Everts and Houtkooper (1975) and Garant and Cho (1984). In contrast to Beertsen, Everts and Houtkooper's study in 1975, only a few fibroblasts were ciliated and, contrary to the findings of Garant and Cho (1984), cilia were not in groups.

Fewer intracellular collagen profiles were noted in the present study than those by Ten Cate and Deporter (1975), Shore and Berkovitz (1979) and Garant and Cho (1979a). Furthermore, specialized intracellular junctions found between rat incisor fibroblasts by Beertsen, Everts and van den Hooff (1974a), and in the rat molar ligament by Frank, Fellinger and Steuer (1976), were not observed. This discrepancy may represent species, age, or regional differences, or may have arisen from the relatively low magnifications used in the present investigation.

Fibroblasts forming a cellular investment around myelinated and unmyelinated nerves, as described by Frank, Fellinger and Steuer (1976) in rat molar periodontal ligament, were also found in the present mouse study. Veil cells formed an incomplete layer around some capillary and postcapillary venule-sized vessels, a finding consistent with that of Rhodin (1968). Descriptions of microfibrils, seen in the present study to occasionally punctuate veil cell cytoplasmic processes have not been found in the literature. Rhodin (1968) reported that veil cells occurred most frequently in the venous circulation. Consequently, the presence of these cells within the ligament, indicates that the periodontal circulation is most probably a venous structure.

b. OSTEOBLASTS

Osteoblasts found in the present study were ultrastructurally similar to those described by Bloom and Fawcett (1975). In contrast to findings by Berkovitz and Shore (1982), an extensive Golgi apparatus was not evident in these cells. However, long cellular processes described by these workers as extending into the alveolar bone towards osteocytes, were found. A comparable description of "satellite" cells situated near the nuclei of osteoblasts that contacted underlying osteocytes has not been found in the literature.

c. CEMENTOBLASTS

Findings by Berkovitz and Shore (1982) that cementoblast morphology is dependent on the functional state of the cell is consistent with findings of the present investigation, where four ultrastructurally dissimilar types of cementoblasts were found. Clearly, further investigation is necessary to differentiate the effect

of plane of section from anatomical variation, before a definitive classification of these cells can be documented.

d. OSTEOCLASTS

Mouse molar periodontal osteoclasts were found to be smaller than human osteoclasts described by Bloom and Fawcett in 1975. Ultrastructurally, the osteoclasts in the present study resembled those described by Bloom and Fawcett (1975) and Berkovitz and Shore (1982).

e. CEMENTOCLASTS

Multinucleated cells and resorption lacunae were not seen on the cemental surface. This is contrary to the findings of Melcher (1976) and may be due either to the age of the animals examined, the sampling technique, or the region of the tooth root investigated.

f. EPITHELIAL CELLS

Ultrastructural features of epithelial cells seen in this study are in close agreement with those detailed in dogs by Owens (1978), and in mice by Heritier and Fernandez (1981). Descriptions by Lester (1969) of the rat molar cell rests of Malassez indicates that the epithelial cells seen in this study were mature.

The number of cells in each cell rest and the juxtaposition of cell rests to the root surface is similar to that described by Melcher in 1976. Distribution of these structures was in accord with that described by Scott and Symons (1982), but was contrary to the findings of Wentz, Weinmann and Schour (1950), and Schour (1960), who examined rat molar ligament.

The fate of epithelial cells within the ligament is the topic of much conjecture, having been discussed by Freeman and Ten Cate (1971), Melcher (1976), Shibata and Stern (1967), Lester (1969), Jande and Belanger (1970) and Spouge (1980). Results from the present study support the concept that whereas some epithelial cells are incorporated into cementum, others degenerate within the ligament. However, further investigation is required to determine the fate of epithelial cells at different depths within the ligament.

g. PERICYTES

In contrast to Rhodin (1968), pericytes within the periodontal ligament exhibited minimal cytoplasmic branching and few membranous contacts with underlying endothelial cells. These cells were otherwise ultrastructurally equivalent to pericytes described by Rhodin in 1968.

h. SCHWANN CELLS

Ultrastructural similarity between mouse periodontal Schwann cells seen in this study, and those described by Griffin and Harris (1968), is evident. No ciliated Schwann cells were found in the present investigation.

i. K-CELLS

K-cells in the mouse molar periodontal ligament were morphologically similar to those in the mouse incisor periodontal ligament described by Everts, Beertsen and van den Hooff (1977), and in rat incisor ligament by Beertsen, Everts and van den Hooff (1974b) and Berkovitz and Shore (1978). However, Everts, Beertsen and van den Hooff (1977) reported the presence of zonulae occludentes between

K-cells but these structures were not found in the mouse molar ligament. Furthermore, fibrillar cross banded structures with a periodicity of between 100 and 170 nm, described by Beertsen, Everts and van den Hooff (1974b) and Berkovitz and Shore (1978), were not observed within K-cells in the mouse molar periodontal ligament. Nevertheless, the low magnifications used in the present study may have precluded their detection.

K-cells were found to be related to mitochondria-rich unmyelinated nerves. In some sections it was apparent that these nerves were in fact nerve terminations, as described by Beertsen, Everts and van den Hooff (1974b) and Everts, Beertsen and van den Hooff (1977). However, on other sections these fibres may have been nerve axons, as described by Berkovitz and Shore in 1978.

The function of these cells is unclear. Although regarded as most probably being proprioceptive cells by Beertsen, Everts and van den Hooff (1974b), these authors also considered that K-cells may assist with tooth eruption. More recently, Berkovitz and Shore (1978) expressed uncertainty as to whether these cells had a sensory function or not. Although K-cell function has not been investigated in the present study, certain ultrastructural features of these cells are of interest. In particular, a well developed Golgi apparatus and a large amount of endoplasmic reticulum and peripheral microvesicles are structures usually associated with secretory cells (Trump and Ericsson, 1965). However, this does not preclude a receptor function for these cells as Higashi, Sasaki, Kawahara and Nakajima (1983) have suggested that the Golgi apparatus may in fact have an absorptive function.

j. MACROPHAGES

Macrophages seen in the present investigation were ultrastructurally similar to periodontal macrophages described by Berkovitz and Shore (1982) and to human gingival macrophages described by Schroeder and Münzel-Pedrazzoli (1973). In contrast to the findings of Berkovitz and Shore (1982) macrophages were the only defence cells found within the ligament. The significance of this has not been investigated.

k. DEGENERATING CELLS

Cells undergoing degenerative changes, described by Wiebkin (1983), were most frequently seen in the middle third of the ligament and were often perivascular in location. The possibility that these profiles represented areas of poor fixation was considered remote because of the excellent fixation of adjacent tissue.

l. COLLAGEN FIBRES

The organization of periodontal collagen was in accord with the descriptions of Melcher (1976), Beertsen and Everts (1979), Barker (1979) and Freeman (1980). An intermediate plexus, reported in the ligament of the continuously erupting rodent incisor by Sloan (1982), or an indifferent fibre plexus, described by Shackleford (1971, 1973), were not evident.

m. OXYTALAN FIBRES

The orientation of oxytalan fibres was similar to that reported by Carmichael (1968) and Sims (1973). The juxtaposition of oxytalan fibres to periodontal cells, reported by Carmichael (1968), was

confirmed by the present study. However, oxytalan fibres were also found within collagen bundles, as reported by Edmunds, Simmons, Cox and Avery (1979), and in close apposition to blood vessels, as described by Sims (1975, 1977). The ultrastructure of fibres seen in the present study is consistent with that described by Sloan (1982).

n. CEMENTICLES

Cementicles, described by Melcher (1976) as calcified masses that may be joined with cementum, were found in the present study only in the apical half of the ligament. They occurred near the cementum surface, cementoblasts and epithelial cells. These structures were considered to represent a calcifying front distant to the root surface.

o. BLOOD VESSELS

Incomplete clearing of blood cells during perfusion was considered indicative of preferential vascular channels within the ligament, as postulated by Folke and Stallard (1967). The orientation and location of periodontal blood vessels seen in this study was similar to qualitative findings by many researchers including, Castelli and Dempster (1965), Kindlová (1965) and Folke and Stallard (1967) in monkeys, Kindlová and Matena (1962) in rats, Corpron, Avery, Morawa and Lee (1976) and Wong (1983) in mice, and Carranza, Itoiz, Cabrini and Dotto (1966) who investigated a number of different laboratory animals.

Blood vessels found in the present investigation could not be categorized on the basis of lumenal diameter and wall morphology in accordance with the classification of Rhodin (1967, 1968). This discrepancy may represent an animal or tissue difference. The most

markedly different vessel type to those described by Rhodin (1967, 1968) were venular-sized vessels, ranging in lumenal diameter from 17 to 26 microns, in which pericytes were sparse or absent.

Sims (1984a) demonstrated that, in longitudinal section, the pericytic investment of capillary-sized vessels is variable and that a given vessel may appear pericytic in one section and apericytic in another. Wong (1983) found, with the scanning electron microscope, that vessels of capillary-size either link apico-occlusally orientated venules, or connect venular vessels with the blood supply of adjacent alveolar bone. It was not possible in the present study to differentiate capillary-sized vessels that interconnected the venular vessels from those that passed through the socket wall. Similarly, because the pericytic investment of vessels is variable, a given vessel may appear pericytic in one section and apericytic in another. Because the different functions of these vessel types has not been ascertained, care must be exercised not to use a pre-existing nomenclature, from which the microphysiology of a vessel can be implied from the name ascribed. A classification of type A vessels has been used for capillary-sized vessels, and type B vessels for the venular-sized ones in which a pericytic cellular investment is sparse or absent. Of these vessel types it was considered that the type A vessels carried the arterial supply and provided interconnecting anastomoses within the ligament. Type B vessels were considered to pass occluso-apically from the alveolar crest to the tooth apex. Clearly, further assessment of vessel ultrastructure is required, so that effects arising from the plane of section can be isolated from anatomical variation and functionally different vessels can be isolated.

Vessel wall and endothelial ultrastructure seen in the present study was consistent with that described by Avery, Corpron, Lee and Morawa (1975) in mice, Frank, Fellinger and Steuer (1976), in rats and Gilchrist (1978) in humans.

## p. LYMPHATIC VESSELS

The absence of vessels, ultrastructurally typical of lymphatic vessels, in this study is in contrast to findings by Levy and Bernick (1968), who examined 20 to 30 micron thick paraffin sections, and Ruben, Prieto-Hernandez, Gott, Kramer and Bloom (1971), who used retrograde carbon perfusion techniques to examine periodontal lymphatics in dogs. Nevertheless, the results of the present investigation are consistent with findings of Frank, Fellinger and Steuer (1976) who examined rat molar ligament using a TEM. This may represent a species difference, or may have arisen from the experimental technique.

## q. NERVE FIBRES

Ultrastructurally, myelinated and unmyelinated nerves found in the present study were consistent with those found by Griffin and Harris (1968), Corpron, Avery, Lee and Cox (1974) and Berkovitz and Shore (1978) who examined human, mouse molar and rat incisor periodontal ligaments, respectively.

The unmyelinated fibres studied in the mouse periodontal ligament varied in diameter from 0.2 to 2.5 microns. This is similar to the diameter of nerves considered by Hannam (1982) to be sympathetic fibres.

Myelinated fibres found in the present study were slightly smaller than those found in human periodontal ligament by Griffin and Harris in 1968. Although Berkovitz and Shore (1978) commented that they could not find evidence that nerves lost their myelin sheaths within the periodontal ligament, such evidence has been obtained from serial sections in the present investigation.

The apico-occlusal orientation of periodontal nerves, reported by van der Sprenkel (1936) in mouse, by Bernick (1956) in rat, by Kubota and Osanai (1977) in the Japanese shrew mole, Bernick and Levy (1968) in marmosets and by Simpson (1966) who examined human tissue, was also found in the present study.

r. NERVE ENDINGS

From the numerous publications about periodontal receptors it is evident that some species specificity of neural receptors occurs (van Steenberghe, 1979; Hannam, 1982). Furthermore, ultrathin sections are inappropriate for delineating nerve endings from fine axons en route to the endings as discussed by Bonnaud, Proust and Vignon (1978). Although this deficiency can be overcome in part by the use of serial sections, descriptions of periodontal receptors from ultrathin sections is problematic. Realizing the limitations imposed by the experimental technique, some findings are worthy of discussion.

The presence of presumptive free nerve endings within the ligament confirms earlier light microscopic work by van der Sprenkel (1936) and TEM investigations by Sims (1983). A lamellated nerve termination located at the junction of two type B vessels and smaller

than that described by Berkovitz, Shore and Moxham (1983) in the rat molar ligament, was found in the present investigation.

K-cells, not previously illustrated in the mouse molar ligament, are considered to possibly be proprioceptors in rodent incisor ligament by Beertsen, Everts and van den Hooff (1974b), Everts, Beertsen and van den Hooff (1974b) and Berkovitz and Shore (1978), and have been discussed previously (section 6.2.i). These results do not support the hypothesis of Cash and Linden (1981) that a single receptor type is present within the periodontal ligament.

Hannam (1982) postulated similarity between cutaneous and periodontal receptors. Capsulated structures, with some anatomical features consistent with Ruffini corpuscles, as described by Halata and Munger (1980), were found in the present mouse study. However, further investigation is required to determine the function of capsulated neural complexes in the periodontal ligament. Similarly, K-cells and cutaneous Merkel cells have some anatomical features in common, but without further investigation, similarity in function cannot be assumed.

## 6.3 STATISTICAL AND STEREOLOGICAL ASSESSMENT

### a. PERIODONTAL CELLS

Beertsen and Everts (1977) studied collagen remodelling in the periodontal ligament of the continuously erupting mouse incisor. They divided the ligament into five zones, four comprising the tooth related part of the ligament, and the fifth being the alveolar compartment, that remained stationary as the tooth erupted. They reported a decrease in the relative volume of fibroblast-like cells from the tooth

surface to the alveolar bone. The volumetric proportion of these cells being 50% in the tooth related part of normal ligament, and 60% in the tooth related ligament of unimpeded teeth. Deporter, Svoboda, Motruk and Howley (1982) investigated areas of extravascular rat molar periodontal ligament, not containing hard tissue or neural elements, and determined that 46.0% of this volume was comprised of cells. Results of the present study indicate a decrease in the number and volume of fibroblast-like cells from the tooth to bone third. However, the volumetric proportion of these cells in the mouse molar ligament was found to be 10 to 15% less than that reported in the rat and mouse incisor ligament.

The volumetric proportion and distribution of epithelial cells has not previously been quantified. The observed frequency of these cells around the tooth root and their juxtaposition to the root surface found in the present study are consistent with qualitative findings by Diab and Stallard (1965) in rats, and Melcher (1976) and Gurling (1982) in mice. However, results of the present study do not support subjective findings, relating to the depth distribution of epithelial cell rests along the tooth root by Wentz, Weinmann and Schour (1950), Schour (1960), Scott and Symons (1982) or Gurling (1982). Neither the absence of these cells in the cervical third of the ligament, nor the biological significance of their distribution has been eludicated from the data collected in the present study.

Pericytes have functions including mechanical support to the blood vessel wall, protection against excessive loss of blood elements, a contractile potential, helping to regulate blood flow (Rhodin, 1968) and proteolysis (Casley-Smith, 1984). Findings of the present

investigation indicate that 88% of the periodontal blood volume is enclosed in vessels, with a mean lumenal diameter of 20.9 microns, in which a pericytic cellular investment is sparse or lacking. Consequently, if functions of mechanical support and prevention of excessive loss of blood elements occur within these vessels, it is probable that other mechanisms are involved. In relation to this concept, the statistically significant correlation between these apericytic venular-sized vessels and oxytalan fibres located in blood vessel walls is of particular interest.

A statistically significant association of K-cells with apericytic type B blood vessels, myelinated and unmyelinated nerves in the body of the periodontal ligament, and oxytalan fibres enclosed in collagen bundles supports the hypothesis that these structures may be functionally interrelated. K-cells were only found at depths greater than 400 microns and then only in the middle and alveolar thirds of the ligament. This may be functionally significant, and requires further investigation.

b. COLLAGEN

Beertsen and Everts (1977), determined that an increase in the relative volume of collagen occurred from the tooth surface to the alveolar bone in the mouse incisor ligament. Whereas only 42.6% of the extravascular ligament nearest the tooth surface was collagen, 62.9% of this volume was collagen in the alveolar compartment of the ligament. Shore and Berkovitz (1979) investigated both rat molar and rat incisor ligament and found that 48.9% of the extravascular portion of the rat molar ligament, was collagen. This is approximately 4% less than the

volumetric proportion of collagen in the tooth related part of the rat incisor ligament. Deporter, Svoboda, Motruk and Howley (1982) investigated areas of extravascular rat molar ligament, not containing hard tissue or neural elements. They determined that 41.9% of this volume was collagen and 12.2% was extracellular space.

A consistent feature of the present mouse molar study with previous rat molar and rat and mouse incisor studies, is an increase in the volumetric proportion of collagen from the tooth surface to alveolar bone. However, the present investigation suggests that the volumetric proportion of collagen is less on the mesial of the mouse molar than in the rat molar or rat and mouse incisor ligament. Although this finding may represent a site difference, the non-random methods of quadrat selection used in the studies by Beertsen and Everts (1977), Shore and Berkovitz (1979) and Deporter, Svoboda, Motruk and Howley (1982), may have biased their results.

The volumetric proportion of collagen in the present study is also in accordance with values derived biochemically, for bovine incisor, by Guis, Shootweg and Tonino (1973) and light microscopically, for human periodontal ligament, by Götze and Kindler (1974).

It is of interest that the volumetric proportion of periodontal collagen and ground substance is less than that in the subcutaneous tissue in rats, where 69.9% of the tissue was collagen and 17.8% ground substance (Casley-Smith and Gaffney, 1981). The functional significance of these structural differences needs to be assessed in order to ascertain the role of principal collagen fibres in tooth support.

c. OXYTALAN FIBRES

A statistically significant correlation between the number of oxytalan fibre bundles enclosed by collagen and the number lying adjacent to periodontal cells suggest that these two fibre groups may represent a single population of oxytalan fibres. However, oxytalan fibres located in blood vessel walls, not being correlated with the other two oxytalan fibre groups, may represent a separate oxytalan fibre population. This hypothesis is consistent with findings of Sims (1977), who reported two distributions of oxytalan fibres within mouse molar ligament. One oxytalan fibre population ran vertically and linked communicating blood vessels. The other extended between blood vessels and cementum. Findings by Sims (1984b), of a statistically significant difference between the microfibrillar proportion of oxytalan fibres adjacent to cells and oxytalan fibres in blood vessel walls, also indicates a dissimilarity between those fibres in blood vessel walls to other fibre groups. The biological importance of these findings cannot be ascertained from the data collected but, if this structural difference is related to a functional one, these findings indicate that oxytalan fibres in the blood vessel walls may be functionally different to those elsewhere in the ligament.

The present findings that 78% of the periodontal oxytalan fibres are located adjacent to cells and of a positive correlation between oxytalan fibres and fibroblasts, suggests a close interrelationship between oxytalan fibres and this cell type. Such findings support the hypothesis that oxytalan fibres may act as a mechanism by which fibroblasts are supported or form a substrata along which periodontal fibroblasts migrate. Alternatively, if fibroblasts

are responsible for the formation and degradation of periodontal oxytalan, then these results suggest that a reasonably high turnover of periodontal oxytalan may occur.

Only three studies have quantified periodontal oxytalan fibres using stereological principles. These are a light microscopic study of human ligament by Jonas and Riede (1980), a TEM study of the rat incisor ligament by Shore, Moxham and Berkovitz (1982), and a TEM study of lathyritic rats by Shore, Berkovitz and Moxham (1984).

Jonas and Riede (1980) calculated changes in stereological parameters of oxytalan fibres in human periodontal ligament following orthodontic treatment. Comparing their results to the present mouse study demonstrates that, although the fibre length per unit volume was approximately 750 times greater in the mouse molar ligament, the volumetric proportion of these fibres was about one seventh that of human tissue. If random orientation to the plane of section is assumed, the mean caliper diameter of individual fibres, quantified by Jonas and Riede in 1980, is 64.4 microns in the control group and 59.3 microns for the orthodontically treated patients. If mathematical correction for a non-random orientation of oxytalan fibres to the plane of section is undertaken then, the mean caliper diameter of these oxytalan fibres is calculated to be 91.0 microns for the controls and 84.2 microns for orthodontically treated subjects. These values are approximately 150 to 300 times greater than those derived by Sims (1981, 1984b), who used a TEM, measured the dimensions of individual transversely sectioned oxytalan fibres and determined that the major and minor axes of human periodontal oxytalan fibres average 0.6 and 0.3 microns respectively. Furthermore, the diameter of oxytalan fibres

calculated by Jonas and Riede (1980) is approximately 10 times that of capillary-sized periodontal blood vessels and approximates the width of the periodontal ligament seen in the present study. Obviously, the stereological data derived by Jonas and Riede (1980) does not represent accurately the anatomical structure of human periodontal ligament oxytalan fibres. This may partly be explained by the limitations of their experimental procedure, including the use of light microscopic techniques, not compensating for tissue shrinkage arising from the use of paraffin sections, assuming that the effect of section thickness could be neglected, and considering random orientation of oxytalan fibres to the plane of section. However, these factors alone would not lead to such misrepresentation of tissue structure and recalculation of the data presented indicates, that rather than a printers error, there had been a basic error in recording the data. Moreover, the stereological data presented was not relevant to conclusions drawn in their publication.

Shore, Moxham and Berkovitz (1982) used TEM techniques to quantify the extravascular regions of the alveolar compartment of the rat incisor ligament in an attempt to describe structural changes when a tooth was relieved from occlusion. Although these researchers did not calculate three dimensional stereological parameters from their findings, performing a stereological analysis on their results (Table 23) demonstrates that the mean caliper diameter of incisor oxytalan fibres approximates mouse molar oxytalan fibres seen in the present study. Furthermore, the oxytalan fibre length per unit volume derived from the data of Shore, Moxham and Berkovitz (1982) is similar to that in the middle, lateral third of mouse molar ligament.

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) |
|---|---|---|---|---|---|---|
| Tooth Third | 0.29 | 1128.6 | 0.3 | 0.6 | 10.1 | 9.4 |
| Middle Third | 0.79 | 2048.2 | 0.4 | 0.7 | 7.5 | 7.0 |
| Bone Third | 0.26 | 577.6 | 0.5 | 0.8 | 14.1 | 13.2 |
| Human Untreated [A'] | 3.02 | 0.5* | 6504.6 | 91.0 | 498.7 | 464.1 |
| Orthodontic Cases [A'] | 4.97 | 0.9* | 5566.4 | 84.2 | 359.6 | 334.6 |
| Impeded Rat Incisor [B] | 0.26 | 2400.0 | 0.1 | 0.4 | 6.9 | 6.5 |
| Unimpeded Rat Incisor [B] | 0.22 | 2260.0 | 0.1 | 0.4 | 7.1 | 6.7 |
| Control Rat Molar [C] | 0.18 | 1780.0 | 0.1 | 0.4 | 8.15 | 7.5 |
| Lathyritic Rat Molar [C] | 0.56 | 2500.0 | 0.2 | 0.5 | 6.8 | 6.3 |

TABLE 23: Stereological parameters for all oxytalan fibres in different zones within the ligament.

A' - Jonas and Riede (1980)
B - Shore, Moxham and Berkovitz (1982)
C - Shore, Berkovitz and Moxham (1984)
* - For the purposes of direct comparison, $L_v$ has been calculated in the same manner as it has for the present study. That is all oxytalan fibres were assumed to be perpendicular to the plane of section. Consequently, the values for Lv appearing in this table are half the value of those in Jonas and Riede's 1980 text. This correction also affects estimation of minimum interfibre distance and mean caliper diameter.

Similarly, Shore, Berkovitz and Moxham (1984) conducted a TEM quantification of ten randomly selected areas of molar and incisor periodontal ligament in normal and lathrytic rats using a digital planimeter. Stereological parameters calculated from the data presented (Table 23) demonstrates a length density of periodontal oxytalan in control molars of 1780 x $10^3$cm/cm$^3$, which is greater than that derived for the total ligament in the present study. In lathyritic animals the length density of periodontal oxytalan was 2500 x $10^3$cm/cm$^3$, a value greater than that found in any region of the ligament in the present mouse molar study. The values derived for mean caliper area are equivalent to those calculated for the rat incisor study by Shore, Moxham and Berkovitz (1982) and are slightly less than values derived in the present mouse molar study. This may represent a species difference, the method by which the sample was selected or recording techniques used.

It is noteworthy that in the studies by Shore, Moxham and Berkovitz (1982), and Shore, Berkovitz and Moxham (1984) only ten areas containing 50 square microns of tissue were quantified. From the results presented it is evident that their data examined, is based on the digitization of no more than 20 oxytalan fibres for each category. In the present study, stereological parameters for oxytalan fibres were based on the quantification of nearly 1,400 oxytalan fibres from 198 quadrats, representing more than 110,000 square microns of tissue. Because it has been found by Sims (1981, 1984b), as well as the present investigation, that variation occurs in the size and distribution of oxytalan fibres within the ligament, the limited sample sizes used by Shore, Moxham and Berkovitz (1982) and Shore, Berkovitz and Moxham

(1984) may have given rise to results that are very much site or sample dependent.

The mean caliper diameter of oxytalan fibres calculated in the present study, to be 0.7 microns, is in excellent agreement with the findings of Sims (1981), who measured the major and minor axes of individual oxytalan fibre bundles in mouse molar ligament. Sims (1981) reported that in the occlusal third, the mean values for the major and minor axes were 1.74 and 0.52 microns respectively. In the intermediate and apical thirds the values for the major axis were 0.84 and 0.57 microns and the minor axes 0.35 and 0.34 microns respectively. A statistical significant difference in fibre size with depth, reported by Sims (1981), was not found in the present study. This may have resulted from either the sampling design used in the present investigation. Furthermore all oxytalan fibres in the present study were assumed to be monodispersed cylinders in a given lateral third of the ligament.

d. BLOOD VESSELS

Gould, Melcher and Brunette (1977) and McCulloch and Melcher (1983) used 1.0 micron thick Epon sections to light microscopically investigate the ligament mesial to the mouse mandibular first molar. They determined that the vascular proportions of the regions they examined were 7.7 ± 0.6% and 7.25 ± 0.75% respectively. Gould, Melcher and Brunette (1977) further found that 73.4% of the vessel volume was in the bone half of the ligament and McCulloch and Melcher (1983) reported that the bone half of the ligament was 4 times more vascular than the tooth half. The percentage vascular volume and the

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | Sv ($x10^3 cm^2/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) | KROGH'S CYLINDER ($\mu m$) |
|---|---|---|---|---|---|---|---|---|
| Total Ligament | 7.46 | 46.5 | 208.8 | 160.3 | 14.3 | 49.8 | 46.3 | 26.1 |
| Human Fat * | | 15.3 | 24.4 | | | | 114 | |
| Human Synovial Membrane * | | 14.1 | 22.5 | | | | 119 | 19.5 |
| Human Knee Tendon * | | 8.5 | 13.5 | | | | 154 | 17.0 |
| Human Synovial Capsule * | | 6.7 | 10.7 | | | | 173 | |

TABLE 24: Comparison of the stereological parameters for blood vessels mesial to the mouse molar with other connective tissues.
* - Casley-Smith, Sims and Harris (1976).

| | $V_v$ (%) | $L_v$ ($x10^3 cm/cm^3$) | Sv ($x10^3 cm^2/cm^3$) | A ($\mu m^2$) | d ($\mu m$) | $ISD^1$ ($\mu m$) | $ISD^2$ ($\mu m$) | KROGH'S CYLINDER ($\mu m$) |
|---|---|---|---|---|---|---|---|---|
| Tooth Third | 0.16 | 2.8 | 7.5 | 58.4 | 13.7 | 204.1 | 189.9 | 107.1 |
| Middle Third | 6.19 | 53.4 | 203.9 | 115.9 | 12.2 | 46.5 | 43.3 | 24.4 |
| Bone Third | 15.78 | 83.2 | 406.4 | 189.3 | 15.5 | 37.2 | 34.6 | 19.5 |
| Mouse Muscle * | | 109.5 | 165 | | | 40.8 | | 17.0 |

TABLE 25: Stereological parameters for blood vessels in different lateral thirds of the ligament mesial to the mouse molar compared with normal mouse muscle.
* - Atherton, Cabric and James, (1984).

distribution of blood vessels across the ligament, derived in this stereological study, are in excellent agreement with those calculated by Gould, Melcher and Brunette (1977) and McCulloch and Melcher (1983).

Sims (1980), who conducted a light microscopic investigation of the ligament surrounding the mandibular first and second molars in mice, determined regional blood vessel volumetric proportions of 17%. Because different areas around the tooth have different vascular volumes (Götze, 1976), the results of the present investigation cannot accurately be compared to those published by Sims in 1980.

The present findings pertaining to vascular volume are also consistent with those by Douvartzidis (1984) who found that the vascular volume of Marmoset monkeys is 8.3%. However, findings by Douvartzidis (1984) that the vascular volume is greatest in the middle, lateral third of the ligament and varied with animal, depth and side of the mouth were not supported by the present mouse study. These discrepancies may have arisen from different experimental techniques or method of sample selection. It may also reflect species and age variation, or vascular anatomy arising from different root number and arrangement.

Wills, Picton and Davies (1976) used various experimental techniques on Macaque monkeys to determine the vascular volume of the ligament. Animals were injected with angiotensin and saline, before exsanguination and subsequent perfusion with formaldehyde. Their results of a vascular proportion of 1 to 2% of the ligament volume are not supported by the present investigation. However, the vascular volume that Wills, Picton and Davies (1976) studied was subsequent to

death by exsanguination, which could cause the vessels to collapse before perfusion with fixative solution.

Findings of the present study are not consistent with those by Götze (1976) who determined that blood vessels occupied only 1.63 to 3.50% of human periodontal ligament and reported a variation in volumetric proportion with depth. Although this may represent a species difference, Sims (1980) has more recently calculated that the regional volumetric proportion of vessels buccal to human premolars as high as 22%.

Stereological investigation of other connective tissues has been conducted by Casley-Smith, Sims and Harris (1976) who investigated human knee tendon and presented stereological parameters for blood vessels in synovial membrane, synovial capsule, fat and tendon. Comparison with their results, presented in Table 24, indicates that the periodontal ligament is approximately 3 to 7 times more vascular than these other connective tissues. However, mouse and human tissue cannot be compared directly because the larger the animal the less its relative vascularity (Casley-Smith, Sims and Harris, 1976). Investigation of human periodontal ligament would allow a more valid comparison of the vasculature of these connective tissues.

Atherton, Cabric and James (1984) have conducted a stereological study of the vasculature of the extensor digitorum longus muscle in normal and dystropic mice. Because their mouse muscle study and the present periodontal ligament investigation used similar methodology and the same assumptions in deriving stereological parameters, direct comparison of the vasculature of these two tissues

is possible. Values calculated for minimum intervessel distance and Krogh's diffusion cylinder demonstrate that the alveolar third of the ligament has a similar vascularity to normal mouse muscle (Table 25).

That the periodontal vascular pool has more than a nutritive function is supported by this study because:

(i) of the disproportionately large vascular volume and vascularity of this tissue compared to other connective tissues;

(ii) the majority the periodontal cells are situated in the tooth third, but this region is the least vascular; and,

(iii) the most vascular region of the periodontal ligament is adjacent to the alveolar bone, the region which statistically, is the least cellular.

The role of the periodontal vasculature in tooth support has been investigated by numerous researchers including Parfitt (1960), Bien and Ayers (1965), Slatter and Picton (1972), Melcher (1976) and Picton and Wills (1978). Although various models have been postulated to explain how this may occur (Bien, 1966; Wills, Picton and Davies, 1976; Walker, 1980), such postulations have been based on assumptions that have little experimental substantiation, or have been based on subjective interpretation. As yet, the method by which the periodontal vasculature supports teeth has not been ascertained. Results from the present investigation provide the first stereological assessment of periodontal blood vessels, from which quantitative comparison with other microvascular beds, where the relationship between structure and function is better understood, can be made.

Vascular responses in the periodontal ligament to tooth loading may involve alteration of regional blood flow within the ligament (Gängler and Merte, 1983). An arrangement of vascular shunts between postcapillary venule-sized vessels, and between these venous vessels and the alveolar bone has been described in the mouse molar ligament by Wong (1983). The finding that pericytes most frequently occurred in these interconnecting vessels supports the hypothesis that these capillary vessels may be the sites where blood flow is regulated. These vessels had a similar length per unit volume to the thin walled venular-sized ones, indicating their prominence within the ligament.

Furthermore, Intaglietta and de Plombe (1973), proposed theoretical concepts of tube-capillaries, where vessel permeability is controlled by the endothelium, and tunnel-capillaries, where it is controlled by the surrounding tissues. Casley-Smith, O'Donoghue and Crocker (1975) investigated jejunal-capillaries in cats and determined that fenestrated capillaries are tunnel-capillaries. Although continuous capillaries behave as tube-capillaries (Casley-Smith, Green, Harris and Wadey, 1975), some tissue regulation of permeability to ions has been demonstrated in continuous vessels by Renkin and Sheehan (1970).

A generalized reference to mouse molar blood vessel ultrastructure has been reported by Avery, Corpron, Lee and Morawa (1975). However, quantification of endothelial microvesicles, junction types and fenestrae is required to determine whether periodontal blood vessels function as tube-capillaries, or whether vessel permeability is regulated by the perivascular connective tissues. It may be possible that periodontal ligament vessels can function as either tube or

tunnel-capillaries under different loading conditions or in different regions along the same vessel. Further stereological and physiological investigation of periodontal blood vessels is required to better understand vascular reactions to normal function changes.

Because changes in the interstitial fluid continuum have also been implicated in the mechanism of tooth support (Bien, 1966; Storey 1973; Wills, Picton and Davies, 1976; Melcher, 1976; Kardos and Simpson, 1979 and Ferrier and Dillon, 1983), the regulation of periodontal blood vessels is of particular interest. If periodontal blood vessels, and in particular the thin walled type B vessels, which contain 88% of the periodontal blood volume, function as tunnel-capillaries, then the mechanisms by which the biophysical properties of perivascular tissue channels are altered, in response to functional loading, is of great significance.

The results of this investigation demonstrate a statistically significant correlation of K-cells and oxytalan fibres with the apericytic type B blood vessels. It is possible, if periodontal vessels function as tunnel-capillaries, that either of these structures may be implicated in the regulation of vessel permeability. However, quantification of vessel ultrastructure is required to determine the possible mechanisms by which capillary filtration rate is controlled and whether or not oxytalan fibres, K-cells and type B vessels are functionally inter-related. Obviously, the mechanism of tooth support is multifactorial and further quantification of the ligament is required in order to correlate structure with function.

e. NERVES

Less quantification has been undertaken for periodontal nerves than for other periodontal structures. Assessment of the number of nerve fibres per unit area was conducted by Kubota and Osanai (1977), who compared the innervation at different depths within the Japanese shrew mole periodontal ligament, using 30 micron thick paraffin sections. These researchers determined that around the mesial root of the lower first molar, the innervation of the apical region was 3 times that of the intermediate zone. This is in excellent agreement with the present investigation, where the same ratios were found. Whereas Kubota and Osanai (1977), reported an average of 4.8 nerve fibres per 1000 square microns of apical tissue examined, an average of 3.6 axons per 1000 square microns of ligament were found in the apical region in the present study. These differences may reflect species differences or the effect of section thickness in the shrew mole investigation.

The smaller mean caliper diameter of periodontal nerves in vessel walls and the lack of statistically significant associations between nerves in blood vessel walls with those elsewhere in the ligament, may not be biologically significant. Nevertheless, it can be postulated from these results that such differences may reflect separate neural populations with different physiological functions. Alternatively, the smaller diameter of axons in blood vessel walls may indicate an abundance of fine nerve endings. Further investigation of these structures is necessary to determine statistical support for these hypotheses. The length density of nerve fibres in blood vessel walls approximates that of nerves elsewhere in the ligament. The significance of a disproportionately large number of unmyelinated

periodontal axons has not been considered, but has been presented in this thesis for completeness.

A comparison of the stereological parameters for nerves in the periodontal ligament with those of nerves in other connective tissues, where the relationship between structure and function is better understood, may well provide additional information relating to the periodontal innervation. Moreover, comparison of the stereological parameters of nerves associated with periodontal blood vessel walls with those associated with blood vessels in other connective tissues, is necessary to ascertain whether or not the innervation of periodontal vasculature is similar to that of other connective tissue microvascular beds.

# CHAPTER 7

## CONCLUSIONS

1. The selected mixture of glutaraldehyde and osmium tetroxide in cacodylate buffer at pH 7.4, delivered at room temperature provided excellent preservation of mouse molar periodontal ligament. The technique of intracardiac perfusion, at the pressures used, neither disrupted vessels nor distorted periodontal tissue.

2. Although the sample size used was small, variation in the volumetric proportion and number of profiles per unit area was not statistically significant between animals. Similarly, these parameters did not vary with depth along the tooth root or between left and right sides of the mouth. However, variation across the ligament from the tooth to the bone thirds was statistically significant for all categories examined.

3. The tooth third of the ligament was the most cellular. Fibroblasts, which constituted 28.4% of the ligament volume, were the most abundant cells within the ligament. Epithelial cells were located in close juxtaposition to the root surface and occurred most frequently at depths in the mid-root region.

4. K-cells, which have not previously been illustrated in mouse molar ligament, had an ultrastructure consistent with active synthetic cells. These cells were found only at depths greater

than 400 microns and then only in the middle and bone thirds of the ligament. K-cells had a statistically significant association with apericytic type B blood vessels, oxytalan fibres enclosed in collagen bundles and nerve axons not located in blood vessel walls. The functional inter-relationship of these structures requires further investigation.

5. Oxytalan fibres were most abundant in the middle lateral third of the ligament and most frequently occurred adjacent to cells, usually fibroblasts. The average length of fibres per unit volume within the ligament was 1251.5 x $10^3$cm/cm$^3$ and their mean caliper diameter, was 0.7 microns.

6. The vascular proportion of the ligament mesial to the mouse molar was 7.46%. The alveolar third of the ligament was the most vascular and few blood vessels were present in the tooth third of the ligament.

7. Two morphological distinct blood vessel types have been found in the periodontal ligament. These comprised type A vessels, that averaged 6.4 microns in diameter, and frequently contained a partial or complete pericytic cellular investment. Type B vessels had a mean lumenal diameter of 20.9 microns and invariably contained few, if any, pericytic cells in the blood vessel wall. These vessels contained 88% of the periodontal blood volume, and comprised 69% and 40% of the blood vessel surface area and blood vessel length, respectively. Further research is required to definitively classify periodontal vessel types. Such classification may well involve

vessel types. Such classification may well involve quantification of endothelial vesicles, fenestrae and junction types. Subsequent physiological studies would then enable vessel structure to be correlated with function.

8. The bone third of the ligament had the greatest neural supply. Approximately 50% of the axons occurred in blood vessel walls and 50% occurred in the parenchyma of the ligament. The correlation between axons within blood vessel walls and those in tissue parenchyma was not statistically significant, indicating that two separate populations of nerves may be present within the ligament.

9. Only 5% of the axons within the ligament were myelinated and these fibres had a mean diameter and length per unit volume of 3.3 microns and 13.7 x $10^3$cm/cm$^3$, respectively. Myelinated fibres seen in the present study were present only at depths greater than 400 microns and were located in the middle and bone thirds of the ligament. By contrast, unmyelinated axons had a mean caliper diameter of 1.4 microns, a length per unit volume of 242.1 x $10^3$cm/cm$^3$, and were present throughout the ligament.

10. Numerous types of neural receptors have been found within the ligament. These comprise free nerve endings, K-cells, lamellated receptors, and encapsulated complexes enclosing at least one myelinated nerve and located adjacent to the vessel lumen of large apericytic vessels. The function and distribution of these receptors has not been ascertained and

11. The stereological parameters determined for the periodontal ligament are dissimilar to those derived for other connective tissues. In particular, the periodontal ligament is seven times more vascular than tendon. Thus the term "periodontal ligament", which infers tissue structure, is probably less suitable to describe this tissue than the term "periodontal attachment", which does not.

12. Although this study provides previously unreported findings pertaining to the ligament mesial to the mouse mandibular molar, it is based on only six teeth from three animals. Although enough sections were quantified to provide a confidence interval of less than ±10% for the material examined, the collection of further baseline data is desirable to ensure that the sample of animals examined is a representative one.

13. Further areas of stereological investigation include the quantification of human periodontal ligament, so that the validity of the mouse molar as an animal model, can be ascertained. To determine physiological changes associated with aging, stereological investigation of older mice would provide invaluable information. Periodontal responses to infection, tooth loading and orthodontic tooth movement similarly require quantification if these processes are to be better understood.

# CHAPTER 8

## APPENDICES

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### 8.1 ANAESTHETIC

Solution: 30% Urethane.

Preparation: 3g Ethyl Carbamate (Urethane) in 10ml of 0.9% Sodium Chloride.

Dosage: 0.1ml/10g body weight.

Shelf life: 2 to 3 days at 4°C.

Route: Intraperitoneal at room temperature.

### 8.2 ANTICOAGULANT

Solution: 1000 I.U. Heparin Sodium in 9ml Ringer's fluid.

Preparation : 90 mg Sodium Nitrate.

1ml Heparin Sodium (1000 units/ml).

9ml Ringer's fluid.

Dosage: 0.02ml/10g of body weight.

Shelf life: 7 days at 4°C.

Route: Intravenous (tail vein) at room temperature.

### 8.3 GLUTARALDEHYDE SOLUTION

Solution: TAAB Glutaraldehyde 25% for electron microscopy.

Preparation: Use stock solution.

Shelf life: 6 months at 4°C.

8.4 CACODYLATE BUFFER

Solution: 0.06M Sodium Cacodylate

Preparation: 25.68g Sodium Cacodylate in 2000ml d.d. water adjust to pH 7.4 using 1N HCl at 20°C.

Shelf life: 7 days at 4°C.

8.5 OSMIUM TETROXIDE SOLUTION

Solution: 4% Osmium Tetroxide.

Preparation: Place 2g of Osmium Tetroxide in 50ml double distilled (d.d.) water. Place the ampoule in hot water to melt the Osmium Tetroxide crystals. Remove from water and rotate the ampoule to allow the melted Osmium Tetroxide to form an even film over the inside. When the Osmium Tetroxide has again solidified remove the label and clean the outside of the ampoule thoroughly with Ethyl alcohol. Then drop the ampoule into a thick walled bottle containing double distilled water and shake to break the ampoule. Wrap in foil to exclude light and leave in a fume cupboard.

Shelf life: 7 to 10 days. This solution can only be used when clear. If it becomes straw coloured, or darker, then its fixative properties are greatly reduced. Refrigeration is not advised as it increases the rate of oxidation and because Osmium Tetroxide is so highly toxic.

8.6 PERFUSATE

Solution: 5.6% Glutaraldehyde and 0.9% Osmium Tetroxide in 0.06M Sodium Cacodylate buffer (final pH 7.4).

Preparation: 7.5ml 0.06M Cacodylate buffer, pH 7.4.

3.0ml 25% Glutaraldehyde.

3.0ml 4% Osmium Tetroxide.

0.54g Dextran 70.

Dissolve Dextran 70 in the 0.06M Cacodylate buffer solution and add Glutaraldehyde. adjust to pH 7.4 using 1N HCl 2 to 3 hours before. Immediately before perfusing add the 4% Osmium Tetroxide solution.

Shelf life: 10 to 15 minutes at room temperature.

Route: Intracardiac via left ventricle.

8.7 DECALCIFYING SOLUTION

Solution: 0.1M EDTA in 2.5% Glutaraldehyde, adjust to pH 6 using 1N HCl.

Preparation: 74.45g EDTA.

1800ml 0.06M Cacodylate buffer pH 7.4.

200ml 25% Glutaraldehyde.

Dissolve EDTA in 0.06M Cacodylate buffer by gentle heating. Cool to 4°C, add Glutaraldehyde, pH to 6.0 at 4°C using 1N HCl.

Shelf life: 7 days at 4°C.

8.8 BLOCK STAIN

Solution: 1% Uranyl Nitrate in 70% alcohol.

Preparation: 1g Uranyl Nitrate.

70ml Ethyl alcohol.

30ml d.d. water.

Shelf life: 7 days at room temperature.

8.9 EMBEDDING MEDIUM (LX-112)

From Ladd Research Industries, Inc.

Because of the nature of the tissue to be sectioned a hard embedding medium was selected.

The weight per epoxide (W.P.E.) of the epoxy resin batch used was 141. Volumetric rather than gravimetric measurements were used although this did not adversely affect the quality of the embedding medium.

Prepartion: For a total volume approximating 130ml.

Mixture A: 28ml DDSA, 22ml LX-112 (shaken vigorously for 10 minutes).

Mixture B: 44ml NMA, 37ml LX112 (shaken vigorously for 10 minutes).

Mixture C: A + B + 1.8ml DMP-30 (shaken vigorously for 10 minutes).

8.10 LIGHT MICROSCOPIC STAINS

(i) 0.05% Toluidine Blue

Solution: 0.05% Toluidine Blue in d.d. water.

Preparation: 0.05g Toluidine Blue.

100ml d.d. water.

Dissolve by stirring.

Shelf life: 6 months at room temperature.

(ii) 1% Borax

Solution: 1% Borax in d.d. water.

Preparation: 1g Sodium Thiosulphate (borax).

100 ml. d.d. water.

Dissolve by stirring.

Shelf life: 6 months at room temperature

8.11 GRID STAINS

(i) 0.5% Uranyl Acetate

Solution: 0.5% Uranyl Acetate in 70% alcohol.

Preparation: 0.125g Uranyl acetate.

7.5ml Ethyl alcohol.

17.5ml d.d. water.

Shelf life: 7 days at room temperature.

8.11 (continued)

(ii) Modified Reynold's Lead

Preparation: (i) 1.33g Lead Nitrate.

1.76g Sodium Citrate.

30ml d.d. water.

(ii) 8ml 1N Sodium Hydroxide

Vigorously shake (i) and allow to stand for 30 minutes, add (ii) then dilute to 50ml with d.d. water mixing by inversion.

Shelf life: 30 days at 4°C. Discard if pH drops below 11.

8.12 RADIOGRAPHIC EQUIPMENT

1. Kodak periapical ultraspeed film 22 x 35mm.

2. Siemens Heliodent machine.

a. accelerating voltage: 50 kV.

b. tube current: 7 mA.

c. exposure time: 0.1 sec.

8.13 TRANSMISSION ELECTRON MICROSCOPE

JEOL 100S (Jeol Ltd., Tokyo, Japan).

1. Accelerating voltage of 60 kV.

2. Beam current of 50 micro-amps.

3. Gun bias setting of 5.

4. Objective lens aperature of 1.

5. Field limiting aperature setting of 2.

8.14 RECORDING SHEET

| | |
|---|---|
| MICROGRAPH NUMBER | |
| ANIMAL NUMBER | |
| SIDE NUMBER (right=1, left=2) | |
| DEPTH (microns) | |
| REGION (tooth=1, middle=2, bone=3) | |

| PROFILE | COARSE GRID | FINE GRID | NUMBER |
|---|---|---|---|
| TOTAL | | | |
| FIBROBLASTS | | | |
| COLLAGEN FIBRES | | | |
| VESSEL LUMEN (i) pericytic capillary | | | |
| (ii) nonpericytic cap | | | |
| (iii) pericytic PCV | | | |
| (iv) nonpericytic PCV | | | |
| (v) other | | | |
| UNMYELINATED NERVES (i) in collagen | | | |
| (ii) in bv walls | | | |
| MYELINATED NERVES (i) in collagen | | | |
| (ii) in bv walls | | | |
| ENDOTHELIAL CELLS | | | |
| PERICYTES | | | |
| VEIL CELLS | | | |
| SCHWANN CELLS | | | |
| K-CELLS | | | |
| EPITHELIAL CELLS | | | |
| MESENCHYMAL CELLS | | | |
| OSTEOPROGENITOR CELLS | | | |
| OSTEOBLASTS | | | |
| OSTEOCLASTS | | | |
| CEMENTOBLASTS | | | |
| MACROPHAGES | | | |
| OTHER CELLS (incl. unknowns) | | | |
| OXYTALAN FIBRES (i) adj to cells | | | |
| (ii) within collagen | | | |
| (iii) in bv walls | | | |
| CEMENTICLES | | | |

## 8.15 STEREOLOGICAL EQUATIONS

1. $V_v = P_p$ (Weibel, 1979)

2. $L_v = N$ (Casley-Smith, 1984)

3. $A = \frac{V_v}{N}$ (Atherton, Cabric and James, 1982)

4. $d = \frac{2 \times (A)^{\frac{1}{2}}}{\pi^{\frac{1}{2}}}$

5. $P = d$ (Atherton, Cabric and James, 1982)

6. $S_v = L_v \times P$ (Atherton, Cabric and James, 1982)

7. $ISD^1 = \frac{1.4142}{(N \times 1.7321)^{\frac{1}{2}}}$ (Atherton, Cabric and James, 1982)

8. $ISD^2 = \frac{1}{(N)^{\frac{1}{2}}}$ (Casley-Smith, 1984)

9. $R = (N \times \pi)^{\frac{1}{2}}$ (Atherton, Cabric and James, 1982)

### SYMBOLS USED IN STEREOLOGICAL EQUATIONS.

| | |
|---|---|
| $P_p$ | Number of test points falling of profiles of a component divided by the total number of test points. |
| N | Number of profiles of a component per unit area. |
| A | Mean profile area of a component. |
| d | Mean caliper diameter of a component. |
| $V_v$ | Volume fraction of a component. |
| $L_v$ | Structure length of a component per unit volume. |
| $S_v$ | Surface area of a component per unit volume. |

| | |
|---|---|
| P | Equivalent diameter of a profile equal in radius to that of the average capillary. |
| $ISD^1$ | Minimum interstructural distance, assuming profiles are situated at the corners of an equilateral trianglular lattice. |
| $ISD^2$ | Minimum interstructural distance, assuming profiles are situated at the corners of a square lattice. |
| R | Radius of Krogh's diffusion cylinder. |

# CHAPTER 9

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ADDENDUM

Programs 7D and 1R of the BMPD-81 statistics package were used for exploratory analysis to isolate which parameters, described in section 5.3 (pages 94-97), should be investigated in further detail. Program 3V was then used to test the components of the general linear model.

The correlation matrices, eluded to in section 5.4 (pages 97-100) and represented in part as Table 6 (page 99), were constructed using program 3D of the BMPD-81 package. This Biomedical Statistical Software Package was produced by The Department of Biostatistics, School of Health, U.C.L.A., Los Angeles, California. It is available from Suite 202, 1964 Westwood Blvd., Los Angeles, California.