5. LITERATURE REVIEW

5.1 Overview of periodontal ligament structure
The periodontal ligament is the connective tissue layer between the cementum covering of the tooth root and the alveolar bone. The ligament forms a link between the tooth and the bone, thus providing support, protection and sensory input for the masticatory system. The structure of the periodontal ligament, like all fibrous connective tissues, comprises a fibrous matrix containing cells, blood vessels and nerves.

In contrast to most connective tissues, the periodontal ligament has a high cellular content with the main cell type, the fibroblast, occupying up to 55 per cent of the ligament in rodents. The fibroblasts within the periodontal ligament lie between the collagen fibres and are shaped like irregular flattened discs. The orientation of these cells is essentially parallel to the collagen fibres, and they are interconnected by numerous gap junctions. Epithelial cells, remnants of Hertwig’s epithelial root sheath, are also present in the periodontal ligament and are seen as small circular aggregates of cells. The unusual feature of the presence of epithelial cells within a connective tissue, and the function of these cells remains the subject of study. This unique periodontal ligament feature will be discussed in detail later. Other cell types present in the ligament include undifferentiated mesenchymal cells and defence cells such as macrophages, eosinophils and mast cells. In addition, the cells lining the hard tissues which delineate the periodontal ligament space, and which are responsible for remodelling of this tissue, are also considered to be part of the cellular population of the ligament. These cells include cementoblasts, osteoblasts, osteoclasts and odontoclasts.

The main fibres of the periodontal ligament are collagen and consist of bundles of cross-banded fibrils. Small amounts of elastic-type fibre, including oxytalan, are also present. The major collagen type found in the periodontal ligament is
type I, with type III also a significant component of the ligament protein, contributing approximately 20 per cent of the collagen. Small amounts of type IV, V, VI and XII have also been detected in the ligament tissue. These different collagen types are important to maintain the normal architectural structure of the ligament and are also involved in regeneration of ligament function associated with remodelling during tooth movement.

The periodontal ligament space can be divided into an inner part related to the tooth root cementum, comprising one-third of the ligament width, and an outer part related to the alveolar bone, occupying the remaining two-thirds. These parts have also been termed avascular and vascular respectively, due to the fact that blood vessels and nerves have been described as running in the outer part, near the alveolar bone. Single nerve fibres, however, have been described in the inner third of the ligament. Most of the nerve fibres which supply the ligament enter through foramina in the alveolar bone near the tooth apex, then run in a coronal direction in bundles parallel to the root surface. Other fibres reach the ligament space through the lateral wall of the alveolar bone, then divide into ascending and descending branches which form a plexus with the nerve fibres originating from the apical region.

5.2 Embryology of dental development
Development of the dental structures begins after thirty seven days of embryonic growth in the human. At this stage, a horseshoe-shaped thickened epithelial band forms in the upper and lower jaw in positions corresponding to the future dental arches. This band of epithelium is known as the primary epithelial band, and gives rise to both the vestibular lamina (which will eventually develop into the vestibular sulcus between the cheek and tooth bearing areas) and the dental lamina, from which the ectodermally-derived tooth components will form. Localised proliferation of cells within the dental lamina gives rise to ingrowths of epithelial tissue at sites corresponding to the future deciduous teeth, and these
ingrowths then proceed through the stages of tooth development – the bud, cap and bell stage – based on histologically identifiable morphological characteristics.

At the cap stage of tooth development, all of the formative structures of the tooth and supporting tissues are identifiable. The epithelial ingrowth, which at this stage has the appearance of a “cap” sitting on a condensation of ectomesenchymal tissue, is known as the enamel or dental organ. This structure is responsible for the formation of enamel, determining the shape of the tooth crown and initiating dentine formation. The condensation of ectomesenchyme is referred to as the dental papilla, which gives rise to the dentine and dental pulp. Surrounding the dental papilla and enamel organ is another condensation of ectomesenchyme known as the dental follicle, from which the supporting structures of the tooth will arise. The overall structure consisting of the enamel organ, dental papilla and follicle is referred to as the tooth germ.

During the bell stage of tooth development, the enamel organ undergoes a process of histodifferentiation whereby four distinct components become evident. The outermost cells of the enamel organ closest to the dental follicle assume a cuboidal shape, forming the external enamel epithelium. Immediately below this layer, the cells synthesise and secrete glycosaminoglycans which lead to increased water content within the enamel organ. This increase in fluid forces the cells apart so that they remain in contact only at their desmosomal junctions, thus giving rise to the star-shaped appearance seen in histologic sections. This morphologic appearance is the basis of the term “stellate reticulum” which is used to describe the layer of the enamel organ immediately beneath the external enamel epithelium. The layer of cells bordering the dental papilla becomes columnar in appearance, and forms the internal enamel epithelium. Between this layer and the stellate reticulum is a layer of flattened cells referred to as the stratum intermedium. The internal enamel epithelium and the stratum intermedium are together responsible for the formation of enamel. The internal and external enamel epithelia meet at the apical border of the enamel organ, with
no interposed cellular layers. The point where this occurs around the rim of the
developing tooth crown is referred to as the cervical loop\textsuperscript{13,14} (Figure 1).

The permanent teeth also arise from the dental lamina, although in contrast to
deciduous teeth, this may occur in two different ways, depending on the
particular tooth type. Permanent incisors, canines and premolars develop from
secondary growth of the original dental lamina of the deciduous tooth, giving rise
to another tooth bud on the lingual aspect of the deciduous tooth enamel organ.
This extension of the original dental lamina, known as the successional lamina,
occurs from the fifth month \textit{in utero} to ten months of age. Once the successional
lamina has formed, tooth development then occurs in the same manner as the
deciduous predecessor. Permanent molar teeth, which have no deciduous
predecessors, form from a distal extension of the dental lamina known as the
accessional lamina. Initiation of the first molar tooth occurs during the fourth
month \textit{in utero}, whereas the second and third molars develop after birth\textsuperscript{13}.

5.3 Development of the tooth root
The development of the supporting structures of the teeth begins with root
formation, which is initiated by the appearance of the epithelial root sheath, first
described by Hertwig\textsuperscript{15} (Figure 2). The epithelial root sheath develops from
proliferation in an apical direction of the internal and external enamel epithelia,
starting at the cervical loop. This proliferation gives rise to a double layer of cells
which grow around the dental papilla, between it and the dental follicle. Hertwig’s
epithelial root sheath determines the final shape of the tooth root. In single-rooted
teeth, the root sheath is a simple collar extending apically from the cervical loop.
In multi-rooted teeth however, a more complex arrangement of the epithelial root
sheath is required. Folds develop in the root sheath and grow inwards towards
each other and fuse, thus forming two or three separate sheaths, depending on
the number of roots the tooth will finally possess\textsuperscript{16}.
Figure 1 Dental embryology

Sagittal section through a developing deciduous incisor at the late bell stage. Note the dental lamina for the permanent successor tooth (DL), internal and external enamel epithelia (IEE & EEE), stellate reticulum (SR), dental follicle (DF) and dental papilla (DP). The cervical loop (CL) is visible at the junction of the IEE and EEE.

(Adapted from Meikle 17)
The cells of Hertwig's epithelial root sheath correspond to the internal and external enamel epithelia of the enamel organ, but without the intervening stellate reticulum and stratum intermedium. Thus the cells of the internal enamel epithelium of the root sheath do not produce enamel. However, they are able to initiate differentiation of neighbouring cells of the dental papilla into odontoblasts which then proceed to produce the dentine of the tooth root. This process of initiation of odontoblast differentiation from dental papilla cells by the internal enamel epithelium also occurs during the earlier development of the tooth crown although in that case deposition of enamel follows the initial dentine formation.

5.4 Epithelial cell rests of Malassez
The epithelial root sheath is rarely observed as a continuous extension from cervical loop to the tooth apex in histologic sections, except in the early stages of root development. In fact, some authors consider that an intact root sheath along the entire developing root is never seen. Once formed, the root sheath initiates root formation rapidly and then breaks up into fragments which form a fenestrated network around the tooth root. The clusters of cells which form this network are known as epithelial cell rests of Malassez (Figure 3). The first sign of degeneration of the root sheath is loss of continuity of the basement membrane, followed by the appearance of collagen fibrils between the epithelial cells. Once the root sheath has fragmented, mesenchymal cells from the dental follicle migrate through it to the surface of the newly formed root dentine. The epithelial cells themselves move towards the dental follicle into the region which will become the periodontal ligament.
Figure 2 Hertwig’s epithelial root sheath
Formation of the root sheath (H) via apical proliferation of the internal and external enamel epithelia. Note the odontoblastic layer (O), predentine (PD), dentine (D), enamel (E) and outer, or external, enamel epithelium (OE).
(Adapted from Furseth 16)

Figure 3 Formation of epithelial rests of Malassez
Breakdown of Hertwig’s root sheath (H) and formation of epithelial rests of Malassez (M). Note the presence of enamel (E), dentine (D), predentine (PD), odontoblasts (O) and external enamel epithelium (OE). Precementum (PC), cementum (C) and cementoblasts (CB) are also visible.
(Adapted from Furseth 16)
The epithelial cell clusters were first fully described by Malassez in 1884, who noted in studies of human teeth that they formed a network around the tooth root. However, it has been reported that the term “epithelial rests” was first used in 1817 by Serres, and that the presence of epithelial cells within the periodontal ligament was identified by several other authors prior to 1884. These early investigators believed that the epithelial cells would atrophy with time and hence be absent in the adult periodontium. Malassez was the first to prove that the epithelial cell rests persisted in the adult periodontal ligament. However, it is currently agreed that the number of epithelial rests does decrease with age, although Spouge suggested that difficulties in accurately distinguishing the epithelial rests within the periodontal ligament tissues may account for variation in reports of their incidence.

5.4.1 Histology of epithelial rests of Malassez

In histologic sections, the network of epithelial sheath remnants is often observed as discrete clusters of epithelial cells which persist within the periodontal ligament into adulthood. Depending on the plane of section, portions of the overall epithelial network are generally observed as small solid circular or oval groups or strands of approximately four to twenty cells. These are usually seen in close proximity to the root cementum, and in some cases may actually be in direct contact with the root surface.

Although it is generally accepted that the epithelial cell rests form a network throughout the periodontal ligament, it is not completely clear as to whether it is a continuous network. Valderhaug and Nylen described an extensive network of epithelial cells which they found to be interconnected upon examination of serial sections. Other authors, however, reported isolated columns of cells or an incomplete network.

The cells of the epithelial rests of Malassez have the typical characteristics of epithelial cells including a surrounding basement membrane which reforms after
the fragmentation of Hertwig’s root sheath. The cells are interconnected by desmosomes, contain tonofilament bundles and very little intracellular material\textsuperscript{1,16}.

In an immunohistochemical study of epithelial rests in the cat, Kvinnsland et al\textsuperscript{25} used stains to detect the presence of neuroendocrine cells. The authors found cells immunoreactive to calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal peptide (VIP). The authors commented that this finding indicated that the epithelial rests are composed of different cell types, in common with epithelia in other locations.

Reitan\textsuperscript{21} noted that there are many variations in the appearance of epithelial rests, but for descriptive purposes classified three typical forms: a pseudo-tubular, round or ovoid form; a cluster-like form; and epithelial strands. All three forms were observed in nearly all cases examined by the author. Reeve and Wentz have described three different types of epithelial cell rests in the periodontal ligaments of humans, namely resting, degenerated and proliferating types\textsuperscript{22}.

\textit{5.4.1.1 Resting type epithelial rests of Malassez}

These cell rests were mainly seen close to the cementum surface, but were occasionally found within marrow spaces of the alveolar bone and beyond the tooth root apex. Both small and large epithelial rests were described, with the former mainly found in younger individuals. These epithelial rests formed strands which appeared oval in cross section and consisted of approximately ten cells. The large epithelial rests generally comprised approximately twenty eight cells which were more loosely arranged and possessed slightly larger nuclei. This type of cell rest appeared in isolated ovoid or spherical clusters and was not seen to form strands\textsuperscript{22}.
5.4.1.2 Degenerated type epithelial rests of Malassez
These were mainly seen in young individuals, and were mostly located in the middle and apical areas of the periodontal ligament, close to the cementum. Each rest comprised approximately ten cells in an oval cluster with a distinct boundary. The cells within the rests were noted to be dark and pyknotic. When present in older persons, these cells rests showed signs of early calcification.

5.4.1.3 Proliferating epithelial rests of Malassez
Located close to the cementum, these epithelial rests were up to ten times larger than the small resting type. Generally found in older individuals, they consisted of cells with large pale nuclei, although great variability in nuclear and cytoplasmic appearance was noted. The cell clusters were often seen to be surrounded by a fibrous capsule which comprised in most cases a concentric arrangement of fibres and cells but sometimes consisted of a hyalinised mass where details of cells and fibres could not be made out.

5.4.2 Ultrastructure of epithelial rests of Malassez
The ultrastructure of the cells of the epithelial rests shows a high nuclear-cytoplasmic ratio; the nuclear outline being irregular with occasional deep infolding. The normal set of intracellular organelles has been reported, with glycogen particles, lipid droplets and microfilaments present, along with relatively abundant mitochondria. A Golgi apparatus and poorly developed rough endoplasmic reticulum have also been described.

Several authors have examined the intracellular contents of the epithelial cell rests at the electron microscope level in order to clarify their role within the periodontal tissues. Hamamoto et al suggested that the epithelial rest cells are inactive, or resting, due to the fact that condensed heterochromatin and a poorly developed nucleolus were seen. Although a Golgi apparatus and vesicles were present, secretory activity of the cells was considered unlikely as the rough endoplasmic reticulum was not well developed. However, it was postulated that...
the cells were able to exchange information, as gap junctions and coated vesicles, which were considered to be a pathway for picking up specific extracellular molecules, were evident. In addition, a primary cilium and associated centriole were often observed. It was concluded that the epithelial rests were more than simple vestigial structures.

In a light and electron microscopic examination of cells from an in vitro culture study of epithelial rests, Yamasaki and Pinero described ultrastructural features of three morphologic cell types.

5.4.2.1 Resting epithelial rests of Malassez
The cells of this type had an ultrastructural appearance similar to that described previously for epithelial rests in vivo. Nuclei were round or ovoid with indentations and peripheral heterochromatin. Mitochondria were present along with poorly developed rough endoplasmic reticulum, and gap junctions between neighbouring cells were evident.

5.4.2.2 Proliferating epithelial rests of Malassez
These cells resembled the basal cells of squamous epithelia. They possessed more abundant cytoplasm and euchromatic nuclei, with free ribosomes and a greater amount of rough endoplasmic reticulum. In the centre of the epithelial islands, cells containing large amounts of tonofilaments were occasionally seen. Desmosomes and gap junctions were not a feature of this cell type. However, microfilament bundles were characteristically seen at the periphery of the cytoplasm. The authors suggested that the structural appearance of these cells was to facilitate cellular motility associated with proliferation.

5.4.2.3 Migrating epithelial rests of Malassez
The authors described two types of cell within this epithelial rest which appeared similar to the basal and prickle cell layers of stratified squamous epithelium. One type consisted of thin cells with inconspicuous organelles and
prominent tonofilaments which were arranged into long, thick fibrils often terminating in well developed desmosomes. The other type comprised thicker cells which contained more organelles and less tonofilaments.

Yamasaki and Pinero 28 discussed the different morphologic types of epithelial rest cell on the basis that these typically inactive cells can be converted to an active state and subsequently proliferate. Suggested stimuli for this conversion include environmental changes such as inflammation within the periodontal ligament 29.

5.4.3 Anatomic location and relationships of epithelial rests of Malassez

As discussed previously, the epithelial rests of Malassez are observed within the periodontal ligament close to the cementum surface 1. Reports vary on the exact distance that the epithelial rests are from the root surface. Distance ranges of 15 – 50 µm 30 and 10 - 100µm 31 have been reported. It has also been reported that the epithelial rests may actually come into direct contact with the cementum surface in some cases 5.

The epithelial rests are found in all regions of the periodontal ligament from apical to marginal regions, but are generally noted to be in the apical area 5. However, age-related changes in epithelial rest distribution have been observed, with Reeve and Wentz 22 reporting that in the first and second decades of life, epithelial rests are more prevalent in the apical third of the periodontal ligament. Later in life, the distribution was more spread out, with 53% found in the cervical third, 26% in the middle third and 21% in the apical third. As mentioned earlier however, it is generally acknowledged that the incidence of epithelial rests within all areas of the periodontal ligament decreases with age 1, 5, 21. It has been reported that the epithelial rests are more frequent on the mesial side of human molar teeth than on the distal side 1 and three to four times more frequent on the mesial root of the mouse first molar than at other sites within the periodontal ligament of this animal 32.
In a study of the relationships between neural structures and epithelial rests in humans, Lambrichts et al. \(^27\) found an intimate association between the basal lamina of the epithelial rests and both free nerve endings and Ruffini-like receptors. A total of twenty-three individual epithelial rests were identified in the material used by these authors (43 teeth), and a close apposition of neural structures to epithelial rests was noted in twenty cases. This close apposition was measured to have a mean value of 0.5\(\mu\)m, and in some cases was up to 30nm. Myelinated nerve fibres were seen to lose their myelin sheaths close to the epithelial rests, which were sometimes completely surrounded by the unmyelinated axons. The authors comment that this high degree of association between epithelial rests and nerves may be significant, especially when contrasted to the moderate density of nerve endings in the rest of the tooth-related part of the periodontal ligament.

### 5.4.4 Functions of the epithelial rests of Malassez

The cells of the epithelial rests are considered by many to be functionless \(^1\). Ten Cate \(^33\) attempted to determine the metabolic pathways used by the rest cells via a histochemical study of enzymes and glycogen within the epithelial rest cells. He found evidence of a metabolism which required little energy and concluded that this both supported the inactive nature of the epithelial rest cells and discounted the possibility of a functional role for these cells within the periodontal ligament.

However several authors have suggested that they represent more than developmental remnants and as such may have a function within the periodontal ligament. As long ago as 1899, Black \(^34\) suggested that the groups of cells which made up the epithelial rests contained a central lumen, indicating a glandular appearance and function. However, the existence of a central lumen and any secretory activity by the epithelial rests has since been disproved \(^5,23\).
5.4.4.1 Maintenance of periodontal ligament space

In a study of tooth replantation, Löe and Waerhaug\textsuperscript{35} noted that ankylosis following replantation did not occur in areas where a vital periodontal ligament containing epithelial rests of Malassez was present, as occurred when the teeth were extracted and then replanted with the ligament tissue intact on the root surface. In cases where the periodontal ligament was allowed to dry or was physically removed prior to replantation of the tooth, initial healing was marked by formation of connective tissue filling the original periodontal ligament space prior to the development of ankylosis. This tissue contained the same cells and fibres as normal periodontal ligament, yet was unable to organise itself into a functional ligament nor maintain its width. Normal periodontal ligament was found around the replanted teeth only when the epithelial rests were preserved. The authors suggested that on the basis of these findings, the epithelial rests may play a role in the maintenance of the periodontal space.

Spouge\textsuperscript{5} comments that bone and cementum are similar tissues and under certain circumstances show a marked tendency to fuse together, yet such fusion does not occur very often, especially given the close anatomical proximity of the two tissues. He goes on to suggest that the presence of epithelial components between the bone and cementum alone may be all that is required to prevent encroachment on the periodontal ligament space by the alveolar bone. The explanation given for this proposition is the known fact that nowhere throughout the body is bone in direct contact with epithelium.

Although suggestions as to the possible role of epithelial rests within the periodontal ligament have been made, these are generally based on circumstantial evidence only, rather than as a result of experiments designed to test the hypothesis. A study by Lindskog et al.\textsuperscript{36}, however, did address this issue. These authors extracted lateral incisors from monkeys and created experimental cavities in the root surfaces of the teeth. Explants of enamel organ epithelium from an unerupted tooth or normal oral squamous epithelium were placed into
the cavities prior to the teeth being replanted. Control teeth were also used in which no epithelial explants of any type were placed into the experimental cavities. Periodontal healing and reparative cementum formation was noted in both experimental groups and the control group; however, in the control and squamous epithelium groups, the alveolar bone grew into the cavities and followed the root surface contour at a similar distance to the normal periodontal ligament width. No traces of explanted oral squamous epithelium were found in cavities originally containing this material, indicating that normal epithelial cells were unable to survive within the connective tissue of the periodontal ligament. In the group where odontogenic epithelium explants had been placed, islands of epithelial cells were observed in the periodontal ligament within the root surface cavities after healing had been completed. The alveolar bone adjacent to these epithelial islands exhibited bay-like resorption indentations, maintaining a space between the bone and the epithelial cells which approximated the width of the periodontal ligament surrounding the tooth root elsewhere. The authors concluded that the odontogenic epithelium used in the study, and hence the epithelial rests of Malassez in the normal ligament, play a role in maintaining the periodontal ligament space.

More recent work on the role of epithelial rests and maintenance of the periodontal ligament space has focussed on the interactions between the epithelial rests and the periodontal ligament innervation. In this study, the authors decreased the amount of epithelial rests in rats by denervating the inferior alveolar nerve, which has been shown to reduce the distribution and size of the rests. After experimental denervation and subsequent reduction in the epithelial rests, ankylosis of the second molar was noted, as evidenced by infra-positioning of this tooth relative to the adjacent teeth. Histologic examination confirmed a narrowing of the periodontal ligament width and areas of direct bone to cementum contact. By ten weeks after the experimental denervation, the epithelial rests were found to have regenerated to a degree, and a corresponding significant increase in periodontal ligament width was noted, although it was
unclear whether the teeth remained in infra-occlusion. The authors concluded that the epithelial rests of Malassez may be involved in maintaining the periodontal ligament width and that sensory innervation may be indirectly involved with this function of the rests.

5.4.4.2 Repair of root resorption
The relationship between epithelial rests of Malassez and orthodontic root resorption has also been considered. Reitan studied the behaviour of the epithelial rests in the periodontal ligament surrounding teeth which had been moved orthodontically. He reported that following the cellular atrophy and subsequent repair associated with hyalinisation of the periodontal tissues, which was commonly seen on the pressure side of the tooth, the epithelial cells did not reappear. Additionally, the author observed that epithelial rests were not present in the periodontal ligament adjacent to areas of root resorption which had occurred during tooth movement. These findings suggested that the epithelial rests are not involved with the regeneration of the periodontal ligament following hyalinisation and orthodontic tooth movement.

In a study of the ultrastructural relationship between epithelial rests of Malassez and orthodontic-related root resorption, Brice et al. observed clusters of epithelial cells within repairing root resorption bays. The cells making up these clusters shared features similar to those described by Yamasaki and Pinero for proliferating epithelial rests. It was further noted that the epithelial cells were only seen in resorption bays which were actually undergoing repair, and not in those areas which showed active root resorption. The authors suggested that these findings may represent a regrowth of epithelial cells into root resorption lacunae and that this may be linked to regeneration of the periodontal ligament and repair of root resorption defects following tooth movement. In support of this hypothesis, the authors discussed the findings of Lester which suggested that cytoplasmic extensions through the basement membrane of the original root sheath of Hertwig may be associated with cementoblast differentiation and the start of
cementogenesis during root formation in the rat. Another study also showed that evidence of cementogenesis in mouse molars was not normally seen until fragmentation of the root sheath, the earliest sign of which was loss of the basement membrane and the appearance of cytoplasmic extensions from the epithelial cells into the surrounding mesenchyme \(^{41}\). Basement membrane discontinuities and cytoplasmic extensions were a feature of the epithelial clusters observed by Brice et al, leading these authors to conclude that the epithelial cells were intimately involved with repair of root resorption \(^{39}\).

In the previously discussed study by Fujiyama et al \(^{37}\), it was also noted that experimental denervation of the inferior alveolar nerve also triggered root resorption in the coronal region of the affected teeth. Increases in odontoclast numbers were noted at the same time as the epithelial rests were disappearing. By ten weeks after denervation, signs of active cementum formation and repair of root resorption defects were seen. This corresponded to the time at which the epithelial rests were starting to regenerate, leading the authors to suggest that the epithelial rests of Malassez may play an inhibitory role in odontoclast appearance and subsequent root resorption, as well as a promoting role in cementum formation and repair of resorption defects \(^{37}\).

5.4.4.3 Development of periodontal ligament innervation

The intimate relationship of the epithelial rests and neural structures within the periodontal ligament has been discussed earlier. By comparison with the Merkel receptor, it has been suggested that this relationship could indicate a target function of the epithelial rests during the development of the innervation of the ligament \(^{27}\). In the case of the Merkel receptor, the epithelial Merkel cell is present prior to nerve fibre contact \(^{42}\), and the budding fibre seeks its target epithelial cell via chemo tropism involving nerve growth factor (NGF) or by simple random searching and recognition \(^{43}\). Thus, it is postulated that the epithelial rests may function in a similar way in the periodontal ligament \(^{27}\).
5.5 Innervation of the periodontal ligament
The innervation of the periodontal ligament provides sensations of pain and touch\(^\text{10}\). Both myelinated and unmyelinated fibres supply the ligament tissue, with the larger fibres responding to forces applied to the tooth and its supporting structures. Smaller diameter nerve fibres are thought to be involved with the reception of noxious stimuli to the periodontal ligament and thus the perception of pain\(^\text{10}\). Receptors within the ligament which respond to force application are known as periodontal ligament mechanoreceptors\(^{44,45}\). Proprioceptive input from the periodontal ligament mechanoreceptors is important in reflex mechanisms, such as those which protect against overloading should a hard object be placed between the teeth\(^\text{16}\). It has also been suggested that the periodontal sensory innervation may interact with immunocompetent cells to assist their migration to inflamed areas of periodontal ligament, for example to take part in the remodelling process during orthodontic tooth movement\(^{46}\).

5.5.1 Anatomy of periodontal ligament innervation
The innervation of the periodontal ligament arises from the trigeminal nerve through either its superior or inferior alveolar branches\(^\text{16,47}\). As described previously, the nerve fibres within the ligament are generally found in the outer part of the ligament space closer to the alveolar bone. A plexus of nerve fibres develops from those that enter the ligament in the apical region and those which perforate the lateral wall of the alveolus. Single nerve fibres, both myelinated and unmyelinated, can be seen branching off from the main nerve bundles and running towards the cementum in the inner part of the ligament. These individual fibres often supply mechanoreceptors within the inner third of the periodontal ligament. Sympathetic nerves have been identified in the ligament, but no evidence of a parasympathetic innervation has been reported\(^\text{10}\).

In a study of nerves in the periodontium of the rat molar tooth using neurofilament peptide immunostaining, Maeda et al\(^\text{48}\) found that few nerves were identified with this antibody in the coronal half of the ligament. These authors described a
dense network of immunopositive nerve fibres in the apical half of the alveolar socket, yet noted that the furcation area contained few such fibres. Sodeyama et al, using PGP 9.5 antibody staining, also reported that the apical region of the periodontal ligament was richly supplied with nerve terminals.

5.5.2 Morphology of periodontal ligament nerve endings
The density of nerve endings within the periodontal ligament follows the same pattern as the nerve fibre distribution, being greatest in the apical region. They can be classified as organised endings, Ruffini or Ruffini-like endings, or free nerve endings.

5.5.2.1 Organised nerve endings
Nerve endings with the appearance of encapsulated corpuscles have been described in the human periodontal ligament. These consist of a central unmyelinated nerve fibre surrounded by a Schwann cell and basal lamina and are considered to be similar to Pacinian corpuscles. This type of nerve ending has not been noted in many other species, however.

5.5.2.2 Ruffini nerve endings
Ruffini terminals are nerve endings consisting of a myelinated axon, its endings and terminal glial cells. Schwann cells surround the nerve ending incompletely, with finger-like projections of the nerve fibre extending into the surrounding connective tissue to contact nearby collagen fibre bundles. A second, smaller type of Ruffini ending has been described, lacking neural finger extensions. The terminal Schwann cells are often seen to partially surround the nerve fibre with multiple cytoplasmic lamellae, and cellular processes also extend into the connective tissue. The location of Ruffini nerve endings has been described as close to the junction of the inner (cementum-related) and middle regions of the periodontal ligament.
5.5.2.3 Free nerve endings

Byers\textsuperscript{11} described four types of nerve ending within the periodontal ligament of the rat, two being of the Ruffini type and the others free nerve endings. The two types of free nerve ending were myelinated and unmyelinated\textsuperscript{11}. Nerve endings of this type have been observed within the periodontal ligament, and they generally contain neurotubules, neurofilaments and vesicles\textsuperscript{10}.

5.5.3 Physiology of periodontal ligament nerve endings

In a study of periodontal ligament mechanoreceptors, Millar et al\textsuperscript{12} were able to locate and mark the position of the individual mechanoreceptors in living experimental animals. After this identification of receptor location, the tissues were processed and examined microscopically. It was found that the only terminal nerve structures found at the marked locations possessed the typical appearance of Ruffini corpuscles. Thus, it is thought that Ruffini endings are solely responsible for mechanoreception within the periodontal ligament\textsuperscript{45}. Periodontal mechanoreception is very sensitive, with detection of forces of only a few grams applied to a tooth and objects of 10-100 µm between the teeth being possible\textsuperscript{16}.

The smaller nerve fibres within the periodontal ligament have been found to respond only to much higher forces and stimuli compared to that required to activate the mechanoreceptors\textsuperscript{45}. This feature, combined with a low conduction velocity of the fibres as well as polymodal response characteristics, suggests that the small myelinated and unmyelinated nerve fibres within the periodontal ligament are involved in nociception\textsuperscript{50}.

Sympathetic innervation of the periodontal ligament is via unmyelinated fibres\textsuperscript{10}. The function of this innervation is thought to be vasomotor, thus affecting regional blood flow\textsuperscript{47}. It is also thought that sensory nerve endings may be involved in blood flow control within the periodontal ligament, via release of
vasoactive substances under mechanical loading\textsuperscript{10}. The sympathetic innervation of the periodontal ligament may also be important in the regulation of cell proliferation in cases of periodontal wound healing\textsuperscript{51}.

5.6 Interactions of epithelial cell rests and periodontal nerves

As has been previously discussed, there is a close anatomical relationship between epithelial rests and nerves within the periodontal ligament\textsuperscript{27}. In a study of the immunoreactivity of rat molar periodontal ligament tissue to tyrosine kinase A (trk A), Yamashiro et al\textsuperscript{38} noted that a staining reaction was found in the epithelial cells of the ligament, and not in any other non-neuronal cells. trk A is a receptor for NGF, which is important for growth and maintenance of sensory and sympathetic nerve fibres. One of the main regions of immunoreactivity of the epithelial rests was the furcation area, and this is a principal region of active remodelling of both the alveolar bone and periodontal ligament\textsuperscript{52}. The findings of Yamashiro et al\textsuperscript{38} lead the authors to propose that the epithelial rests, in association with the periodontal ligament nerves, may be involved in alveolar bone remodelling.

The second part of the study by Yamashiro et al\textsuperscript{38} involved disrupting the innervation to the periodontal sensory nerve endings by transecting the inferior alveolar nerve. The authors noted that this intervention caused a decrease in the number of epithelial islands in the periodontal ligament within one week, and this continued up to three weeks. The sizes of the epithelial rests were also seen to reduce. On the basis of this finding, together with the previously reported close association between epithelial rests and periodontal nerves\textsuperscript{27}, the authors suggested that the sensory innervation may play a role in maintaining the epithelial rests of Malassez\textsuperscript{38}.

In another study involving trkA immunoreactivity in the periodontal ligament, Woodnutt and Byers\textsuperscript{53} noted that all epithelial rests which were identifiable via light microscopy showed definite trkA immunoreactivity. This reactivity was
observed mainly around the periphery of the epithelial cell clusters. However, a high degree of association between epithelial rests and periodontal nerve fibres was not seen. The authors concluded that NGF binding to the trkA receptors on the epithelial cells could act as a means of communication between the epithelial rests and the periodontal ligament environment, particularly as trkA expression was at the periphery of the epithelial rests rather than all through the cell clusters. It was suggested that this epithelial rest – periodontal ligament interaction may be unrelated to innervation 53.

5.7 Dentoalveolar Ankylosis
Dental ankylosis is defined as fusion of the cementum with alveolar bone 54. Ankylosis is most common in deciduous molar teeth, although it can also occur in permanent teeth, with the first molar being the most likely affected tooth 55. It has also been reported that the incidence of ankylosis is twice as frequent in the mandible as the maxilla 56.

Ankylosis of a tooth stops its eruptive potential, leading to infra-occlusion of the affected tooth due to continued eruption of neighbouring teeth. Thus, ankylosis can be considered a disturbance of eruption. Biederman 54 defined three potential causes of cessation of eruption, these being physical obstruction (i.e. impaction), destruction or defect of the dental papilla and ankylosis. A more recent differential diagnosis system classifies arrested eruption as impaction, primary retention or secondary retention 57. These categories correspond in aetiology to the respective definitions as described by Biederman 54, with the additional qualification that primary retention refers to cessation of eruption prior to emergence of the tooth into the mouth and after emergence for secondary retention.

5.7.1 Aetiology of ankylosis
The periodontal ligament is interposed between the alveolar bone and tooth root, so for ankylosis to occur, a discontinuity in the ligament must be present. This
may arise due to incomplete development of the ligament or as a result of local lysis. Alternatively, direct local ossification of the ligament tissue may occur, although this was not considered as a likely event by early authors in the field. More recent work has noted that calcifications may develop within the hyalinised areas of the periodontal ligament during orthodontic tooth movement. Further study showed that calcification of the degenerative tissue of the hyalinised zone began within the first day of tooth movement. The authors proposed that calcification of this type within the periodontal ligament may actually be a protective response to prevent direct contact between cementum and bone, which would lead to ankylosis.

Given the fact that, for ankylosis to occur, it appears that a gap must develop in the periodontal ligament, three causes of dental ankylosis were suggested by Biederman.

5.7.1.1 Congenital gaps in the periodontal ligament
This condition could realistically only be an explanation for primary retention, but ankylosed unemergent teeth are considered to be rare.

5.7.1.2 Local periodontal ligament trauma
This theory suggests that local injury to the periodontal ligament, followed by ossification during the healing process may lead to ankylosis. Early experimental work could not produce ankylosis via direct pressure or trauma, although success was achieved using extraction and replantation. Thus trauma and excessive pressure were not considered to be likely causes of ankylosis. However, traumatic injuries to the teeth which result in a defective periodontal ligament are currently known to cause ankylosis.

5.7.1.3 Disturbed local metabolism
Prior to exfoliation of deciduous teeth, resorption of the root occurs first, followed by disappearance of the periodontal ligament. However, should the ligament
disappear before the root has resorbed sufficiently, the cementum and alveolar bone could potentially come into contact and thus lead to ankylosis. The aetiology of ankylosis is also discussed by Raghoebet al. These authors discuss the possibility of a disturbance of the interaction between normal root resorption and hard tissue repair as a potential cause of ankylosis. In their study of secondary retention of permanent molars, physiologic local root resorption was occasionally observed in normal teeth. This resorption was repaired by new cementum formation and the root shape was re-contoured. It was suggested that a disturbance of this repair process could occur whereby the usual cementoblasts are replaced by osteoblasts, with osteoid material being deposited within the resorption lacunae and the possible development of ankylosis. It was also pointed out that molars usually exhibit the largest number of resorption areas, which may explain the preponderance of ankylosis in this tooth type compared to all other permanent teeth. The authors conclude that a developmental problem within the periodontal ligament may be the reason this type of ankylosis occurs, and to support this contention they cite the fact that a familial tendency for ankylosis has been reported.

Teeth which have been traumatised, particularly if they have been avulsed or luxated, have a high incidence of ankylosis. Andreasen has reported that root resorption is a potential late complication following dental luxation injuries, with external root resorption much more common than internal resorption. External root resorption can be classified into three types: surface, inflammatory and replacement resorption. The latter type is characterised by direct contact between bone and tooth root, with gradual replacement of tooth hard tissue by bone. The author uses the term replacement resorption when describing the condition of ankylosis.
Homeostasis between the periodontal ligament fibroblasts and the bone cells lining the inner aspect of the alveolus has been proposed as one of the ways that the width of the ligament may be maintained. It has been suggested that the cells of the periodontal ligament are able to inhibit osteogenesis, thus preventing ankylosis. When the homeostasis between the periodontal ligament cells and bone cells is interfered with, ankylosis results. This was accomplished in one study via the administration of the drug 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP). The possible actions of this drug include inhibition of bone resorption, an increase in bone matrix formation, and a cytotoxic effect on the periodontal ligament fibroblasts. When HEBP was administered to experimental rats, a significant decrease in periodontal ligament width was noted, with ankylosis evident after thirty days.

Finally, temporary or permanent disruption of the nerve supply to a particular oral region has been suggested as a possible cause of primary and secondary retention (ankylosis) of permanent teeth. Disruptions of this sort may be associated with herpes zoster and mumps infections, with spread of the virus along nerve branches. This hypothesis was suggested particularly in cases where more than one permanent tooth exhibited primary or secondary retention.

5.7.2 Diagnosis of ankylosis
In a clinical and histologic study of secondary retention of permanent molars, it was noted that clinical and radiographic signs of ankylosis matched the histologic findings in only six out of twenty-six cases. Percussion testing was found to be more accurate than radiographs, especially for multi-rooted teeth. It was found that clinical tests often provided false negative results for ankylosis but rarely false positives. Infraocclusion was recommended as the most reliable clinical criterion in the diagnosis of ankylosis. The clinical sign of a tooth appearing submerged once initially having reached the level of the occlusion has in fact been referred to as pathognomonic of ankylosis.
5.7.3 Histological appearance of ankylosis
The principal histologic finding in an ankylosed tooth is direct contact between the alveolar bone and root surface without any intervening periodontal ligament. However, studies have shown that the area of contact between root and bone is usually no more than 10-60% of the root surface of the affected tooth. In this study of secondary retention of molar teeth, areas of ankylosis were most commonly observed in the furcation and interradicular regions of the teeth and occasionally at the apical region. Hypercementosis with direct bone contact was sometimes seen in ankylosed teeth, particularly in the apical area. Sometimes bridges of bone were seen in direct contact with the dentine itself, and resorption lacunae were present. Hypercementosis was also observed adjacent to the bony bridges.

5.7.4 Treatment of ankylosis
Biederman, in 1956, recommended attempting to free an ankylosed tooth by slightly rocking or luxating it, with the aim being to break the points of fusion between cementum and bone in the hope that fibrous tissue would intervene and lead to the reformation of a normal periodontal ligament. Later guidelines published by the same authors suggested a range of management options. These options were: immediate extraction, building up occlusal and proximal contacts, luxation, or leaving the affected tooth undisturbed. The options of leaving the tooth and occlusal build up were suggested in cases where ankylosis had occurred close to or after maturity. Extraction was indicated for ankylosed deciduous teeth where the permanent successor is present, and luxation was recommended for the ankylosed permanent tooth.

However, Raghoebert et al were of the opinion that luxation may not be an effective treatment modality for the ankylosed tooth on the basis of their findings that the site of bone-cement fusion was often the furcation region. They explained that ankylosic zones in this area are very difficult to break via luxation.
of the tooth as the centre of rotation during luxation is at the furcation. They believed that luxation may in fact promote further ankylosis rather than eliminate it. These authors presented a management approach based on the timing of ankylosis relative to the growth of the patient. Should ankylosis be apparent prior to the growth spurt, immediate extraction followed by orthodontic space closure was recommended. During the growth spurt, observation was suggested, with extraction indicated if infraocclusion is seen to be progressive. After the growth spurt, prosthetic build up of the tooth was suggested in cases where infraocclusion was minor and extraction with possible prosthetic replacement if infraocclusion was severe.

A recent study \(^{67}\) presented several other treatment possibilities for ankylosed permanent teeth, with two surgical approaches described in addition to the options discussed above. These options were interalveolar segmental osteotomy and localised ostectomy. The first option involved surgically repositioning the intact ankylosed tooth and a thin section of surrounding bone, whereas the second involved surgically eliminating the area of ankylotic bone fusion via flap surgery and curettage of the affected osseous material. The latter alternative was presented as a practical option only when the ankylotic region is located in the crestal area and is readily definable and accessible. The authors also suggested that luxation of the affected tooth may be a viable treatment modality, provided a prolonged heavy orthodontic extrusive force is applied to the tooth immediately after surgical luxation. A case report was presented in which surgical luxation and extrusion via lingual orthodontics was successful in treating an ankylosed permanent molar in an adult patient.

5.8 Experimentally induced ankylosis
Several methods of inducing ankylosis in experimental animals have been reported in the literature, thus allowing detailed study into the causes, histology and pathogenesis of this condition. The common factor in these methods is the
production of some kind of damage to the periodontal ligament tissue, whether direct or indirect.

5.8.1 Trauma
Parker et al.\textsuperscript{68}, in a radiographic and histologic study on dogs, were able to induce ankylosis by mechanically injuring the tooth root and periodontal tissues and then removing the teeth from occlusion and splinting them. However, ankylosis was not confirmed histologically in every case, even when radiographic evidence suggested it was present. However, when a similar protocol was used on monkey teeth in another study, no evidence of ankylosis was found.\textsuperscript{69} These authors were able to produce ankylosis by luxating the tooth to the point that it was mobile in all directions whilst still remaining within its socket.

5.8.2 Extraction and replantation
Several tissue reactions have been described following experimental extraction and replantation of teeth in experimental animals.\textsuperscript{70} These include: no root resorption, root resorption with subsequent cemental repair, active inflammatory resorption, and ankylosis. Carrying out endodontic treatment on the extracted teeth prior to replantation prevented inflammatory resorption.\textsuperscript{71}, so that the tissue response seen after replantation was related to the extent of tissue damage and cell death in the periodontal ligament of the extracted tooth. Small areas usually showed repair whereas larger areas of periodontal damage progressed to ankylosis.

5.8.3 Pharmacological and chemical models
As discussed earlier, Wesselink and Beertsen\textsuperscript{63} were able to produce experimental ankylosis though the administration of the drug HEBP. The mechanism of this protocol was thought to be a disruption of the normal homeostasis between the periodontal ligament cells and those lining the alveolar bone of the tooth socket.
The production of ankylosis via chemical injury to the periodontal ligament has been reported \(^6^9\). These authors cite work by Gottlieb and Orban in which treatment of the root canal with formalin was used to induce ankylosis.

### 5.8.4 Disruption of innervation

Berggreen et al \(^7^2\) examined the influence of the sensory innervation on periodontal healing following extraction and replantation. In an experiment using ferrets, the authors produced denervation via axotomy of the inferior alveolar nerve on one side, with the opposite side serving as a control. The lower first premolars were then extracted and replanted. Histologic examination revealed that resorption of the roots of replanted teeth was greater on the innervated side, but the incidence of ankylosis was similar whether innervation had been disrupted or not. The authors concluded that the sensory innervation may promote root resorption after pulpo-periodontal injuries but have less influence on the osteoblastic activity associated with ankylosis.

In a more recent study by Fujiyama et al \(^3^7\), however, ankylosis was reliably produced after transection of the inferior alveolar nerve in rats. The ankylosis, which was observed after six weeks, was found in the coronal region of the periodontal ligament, and it was also noted that root resorption was activated after denervation. Histomorphometric data from this study suggested that denervation activated bone formation at the alveolar bone surface, consequently resulting in ankylosis.

### 5.8.5 Thermal injury

Rubin et al \(^6^9\) discussed the use, as long ago as 1930 by Gottlieb and Orban, of electric diathermy as a means of inducing experimental ankylosis in dogs. However, more recent methods have focussed on the use of a cold stimulus to produce ankylosis.
Wesselink et al \(^{73}\) applied liquid nitrogen to the outer surface of the lower jaw of the mouse in order to freeze the periodontal tissues of the incisor tooth. When light and electron microscopic examination was carried out after this treatment, cell death within the periodontal tissue was noted. After approximately one week, resorption of the tooth root was observed along with some ankylosis.

A similar technique was also used by Tal and Stahl \(^{74}\) with rats as the experimental animal. These authors reflected the gingival tissues over the buccal aspect of the first molar teeth, then applied a cryoprobe capable of generating temperatures of -81°C directly to the exposed bone surface. Marked root resorption and reparative cementum were noted after five to seven weeks, as were areas of ankylosis.

Dreyer et al \(^{75}\) refined the earlier methods of thermal insult using a cold stimulus in order to limit the associated injury to the periodontal ligament rather than the surrounding tissues. The technique developed by these authors consisted of applying pellets of solid carbon dioxide (dry ice) to first molar crowns of rats. Application of the cold stimulus was continuous for a period of ten or twenty minutes, with a second group of animals subjected to three episodes of freezing. A further group also underwent mechanical trauma to the periodontal ligament. When studied histologically two days after freezing, the teeth showed minor root resorption near the apex with only mild signs of fibre disorganisation and hyalinisation apparent within the periodontal ligament. By seven days, shallow resorption lacunae, localised to the cervical and interradicular areas, were observed with associated multinucleated cells. Marked periodontal ligament disorganisation and extensive areas of hyalinisation were noted. After fourteen days, the resorption lacunae were larger although the multinucleated cells had reduced in number and signs of cementum repair were present. Active bone resorption was occurring at this stage. By twenty eight days, active root resorption had ceased and repair of the lacunae was progressing.
The results of this study indicated that a longer freezing time, multiple freezing episodes and additional mechanical trauma generated more extensive injuries to the periodontal tissues. The tissue responses in the group with a single, short freezing episode were dominated by the root resorption and repair processes described above. However, in the groups which received multiple freezing episodes or single long episodes, ankylosis was often noted in the interradicular area and at times was quite widespread. The localisation of ankylosis in the interradicular region was suggested to be related to the difference in thermal conductivity when a stimulus is applied parallel or transverse to the dentinal tubules, as the alignment of dentinal tubules in the interradicular area paralleled the direction of application of the cold stimulus. The development of ankylosis was explained as possibly an effusive reparative response by the tooth and alveolar bone following periodontal tissue destruction by the freezing treatment.

Recently, the protocol of Dreyer et al. has been used, with a single twenty minute freezing episode, in a study of dentoalveolar ankylosis. In this investigation, similar tissue reactions were reported. At seven days after freezing, shallow resorption lacunae were seen along with some disorientation and hyalinisation within the periodontal ligament. These changes were more marked by fourteen days, and ankylosis was observed at this time. By twenty eight days, root resorption had ceased although ankylosis was still present. Repair of resorption occurred between fifty six and eighty six days, and ankylosis was noted to be widespread during this period.

5.9 Histologic methods for investigations of the epithelial rests of Malassez
Early investigations of the epithelial cell rests within the periodontal ligament used light microscopy and haematoxylin and eosin staining. This staining protocol has also been used in more recent studies. Another early study by Ten Cate used histochemical techniques to investigate enzymes within the cells of the epithelial rests. Nitro blue tetrazolium was used in that study, with the
fresh sections incubated in the substrate solution prior to fixation in formalin and counterstaining with methyl green.

5.9.1 Immunohistochemistry

Immunohistochemistry is a technique whereby antibodies are used to detect and locate specific antigens within a particular tissue. One of the main advantages of this technique is its specificity, as the target antigenic molecules are restricted to certain cell types. The antigen is generally a macromolecule, and the antibody raised against it binds to a small area of the molecule known as an epitope. Immunohistochemical techniques have been used by a number of investigators in the study of epithelial cell rests.

5.9.1.1 Keratins and cytokeratins

These two interchangeable terms relate to a group of water-insoluble fibrous proteins which constitute tonofilaments in most epithelial cells. These cytoplasmic proteins are also known as intermediate filaments and are major components of the cytoskeleton, forming fibrillar arrays between the membrane and nucleus of the cell. They may serve to anchor the nucleus and organelles within the cell. There are more than twenty different cytokeratin proteins in human epithelial cells, and different sets of these polypeptides are synthesised in different epithelia. Thus, a specific pattern of cytokeratins characterises each given type of epithelial tissue.

Several studies have used antibodies to specific cytokeratins for investigation of the epithelial rests of Malassez. Gao et al. observed a positive staining reaction to cytokeratin 5 and 19 in human material. Peters et al. using rabbit tissue, noted immunohistochemical detection of cytokeratin 5, 7, 8, 14, 15, 17, 18 and 19 although it was noted that staining with antibodies to cytokeratin 18 was very weak. Berkovitz et al. found staining for cytokeratin 5, 6, 8, 17 and 19 in bovine tissue. The variance in the results of these studies may be due to the different species used for collection of periodontal tissue, although cytokeratin 5, and 19
were found in all three studies, and possibly cytokeratin 17 also, as this was not included in the study by Gao et al.\textsuperscript{82}

A combination of two monoclonal antibodies, AE1 and AE3, have been shown to recognise almost all keratins when used together\textsuperscript{78, 79}. This antibody combination was successfully used in a recent investigation of epithelial rests of Malassez in the periodontal ligament of the rat\textsuperscript{84}.

5.9.1.2 Trk A

Several authors have used anti-trkA antibodies to identify epithelial rests in the periodontal ligament. Yamashiro et al.\textsuperscript{38} used both 50µm cryostat sections and 7µm paraffin embedded sections for immunohistochemistry of rat periodontal tissue. They used rabbit anti-trkA antibody to identify epithelial rests and found that immunoreactivity was not seen in any other non-neuronal cells within the periodontal ligament. Neural tissue also did not exhibit immunoreactivity. On the basis of these findings, the authors suggested that trkA may be a useful marker for epithelial rests within the periodontal ligament. The same antibody (sc-118, Santa Cruz Biotechnology, Santa Cruz, California) was also used as a marker for epithelial rest cells by Fujiyama et al.\textsuperscript{37}, but these authors only used 50µm cryostat sections for immunohistochemistry.

Woodnutt and Byers\textsuperscript{53} found immunoreactivity for a truncated 41kDa form of trkA in the epithelial rests of rat periodontal ligament. They used an antibody (sTA, Santa Cruz Biotechnology, Santa Cruz, California) which demonstrated specificity to this truncated form of trkA, and noted that the epithelial rests showed intense immunoreactivity. Floating-section immunocytochemistry and immunofluorescence techniques with 40µm sections were used in that study.

5.10 Detection of nerves within the periodontal ligament

A number of techniques have been used to study the innervation of the periodontal ligament. Early studies, for example by Bernick in 1959\textsuperscript{85}, used silver
impregnation methods which yielded valuable information but also caused some misinterpretations due to non-specific staining and difficulties with staining calcified tissue \(^{48}\). Byers \(^{11}\) used light microscopic autoradiography to study periodontal ligament nerve endings. Labelling isotopes were injected into rats and were used to map nerve endings via axonal transport.

5.10.1 Neurofilament proteins

More recently, however, immunohistochemical techniques using antibodies to a variety of neural components have become more popular. Antibodies to neurofilament protein have been used by several investigators \(^{48, 86, 87}\). Neurofilament protein makes up the neuronal cytoskeleton and is abundantly present in most neurons. Most of the studies cited used frozen sections for the immunohistochemical procedures, although Luukko et al \(^{87}\) used paraffin embedded sections. These authors employed *in situ* hybridisation to study the expression of neurofilament light-chain mRNAs.

5.10.2 Protein gene product 9.5

Protein gene product 9.5 (PGP 9.5) and neurone specific enolase (NSE) have been suggested as general markers for nerves and neuroendocrine cells \(^{88-90}\). NSE is an isoenzyme of the glycolytic enzyme enolase, and is exclusively localised in mammalian neurones as well as central and peripheral neuroendocrine cells \(^{88}\). NSE was used by Fristad et al \(^{89}\) to allow comparison between nerves which showed immunoreactivity to specific peptides and the general innervation of the developing rat molar and its supporting tissues. PGP 9.5 is a soluble protein isolated from human brain, and is a major component of the cytoplasm of neurons \(^{91}\).

Ramieri et al \(^{91}\) were the first investigators to use PGP 9.5 to study the innervation of teeth and oral tissues. They reported that PGP 9.5 appears to be more generally expressed than neurofilaments in fine nerves, with both neurofilament and NSE markers failing to demonstrate small nerve endings in
peripheral tissue. The results of their study showed that the neuron-specific PGP 9.5 marker was also expressed in small nerve endings and corpuscles. The authors concluded that PGP 9.5 is a reliable marker for the demonstration of fine nerve terminals in human gingiva and pulpal tissue. PGP 9.5 was also used in studies of periodontal ligament innervation in rats and mice \(^90, 92\) and in studies of the innervation of developing human \(^93\) and rat \(^94\) teeth using paraffin embedded sections.
6. RATIONALE OF THE CURRENT STUDY

The maintenance of the periodontal ligament space and thus prevention of dentoalveolar ankylosis has been suggested as a possible role of the epithelial rests of Malassez \(^{5, 35-37}\). Studies have shown that the epithelial rests display a close anatomic relationship with nerve endings within the periodontal ligament \(^{27}\), and disruption of the innervation of the ligament was found to be associated with a decrease in the size and distribution of the epithelial rests \(^{38}\). These findings, when considered together, suggest an interactive function of the epithelial rests of Malassez and innervation in the maintenance of the periodontal ligament.

The aetiology of dentoalveolar ankylosis is still not well understood. Disturbance of local metabolism \(^{54}\) or of the homeostasis between various periodontal ligament tissues \(^{63}\) have been suggested as possible causative factors. As discussed above, both the epithelial rests of Malassez and the periodontal innervation may be involved in maintenance of the ligament. Disruption of the innervation has also been implicated as an aetiologic factor for ankylosis \(^{65}\), and experimental denervation has been reported to produce this condition \(^{37}\).

On the basis of these possible relationships, it is intended in the current study to examine the distribution of the epithelial rests of Malassez and the nerve fibres of the periodontal ligament in teeth in which ankylosis has been experimentally induced. This may allow further elucidation of their combined role in the development of ankylosis or protection against it. Ankylosis will be induced using the thermal injury protocol of Dreyer et al \(^{75}\), which has been shown in a recent study to reliably produce ankylosis in rat molar teeth \(^{76}\). The epithelial rests will be identified immunohistochemically using cytokeratin (AE1, AE3) markers which have previously been found to identify these structures on paraffin embedded sections of rat periodontal ligament tissue \(^{38, 84}\). Nerve fibres will be identified using PGP 9.5 markers, as has been reported in earlier reports in the literature \(^{87, 94}\). Rats will be used in this experiment due to the common usage of this animal
for periodontal ligament studies, its ease of handling and the fact that the thermal injury model to be used was developed and refined using rats as the experimental animal \textsuperscript{75, 95}.
The aims of this study are:

1. To investigate the distribution of epithelial rests of Malassez and neurofilaments within the periodontal ligament.

2. To examine the response of the epithelial cell rests of Malassez to aseptic periodontal necrosis caused by hypothermic injury, and to determine their influence on the development of ankylosis and root resorption.

3. To examine the response of nerve fibres to hypothermic periodontal injury and to investigate any interrelationships with epithelial cells.

7.1 Null hypotheses

7.1.1 Epithelial rests and ankylosis
Epithelial rests of Malassez do not provide a protective function against ankylosis and external root resorption.

7.1.2 Nerve fibres and epithelial rests
Nerve fibres and epithelial cells are not inter-dependent.
8. MATERIALS AND METHODS

8.1 Experimental animals
30 eight week old male Sprague Dawley rats were obtained and randomly divided into six groups of five animals. All animals were housed in the Animal House facility of the Medical School of the University of Adelaide and were fed a diet of commercially manufactured standard rodent pellets (Parastoc Feed, Ridley AgriProducts, Murray Bridge, Australia) and water, ad libitum. Approval of the experimental procedures was provided by the Ethics Committee of The University of Adelaide under ethics number M-01-2004.

8.2 Anaesthesia
The rats were anaesthetised prior to the experimental procedures to render them unconscious and to prevent reflex activity. For this purpose, a combination of Hypnorm® (fentanyl citrate, 0.315 mg/ml and fluanisone 10 mg/ml; Janssen-Cilag Ltd., High Wycombe, Buckinghamshire, UK) and Hypnovel® (midazolam hydrochloride, 5 mg/ml; Roche, Berne, Switzerland) was used. The two drugs were each diluted 1:1 with sterile water for injection, combined, and then administered intramuscularly at a dosage of 2.7 ml/kg of body weight. This method provided rapid and predictable anaesthesia of approximately 2 hours duration, allowing the experimental procedures to be carried out with minimal discomfort to the animals. In addition, there was adequate time for the frozen tissues to thaw prior to the animal regaining consciousness. Onset of sufficiently deep anaesthesia was confirmed prior to experimental procedures or sacrifice by checking for the absence of both the plantar and corneal reflexes.

For animal sacrifice and harvesting of tissue, an alternate anaesthetic agent was employed due to the fact that the available supplies of Hypnorm® were low and this drug was no longer being manufactured. Nembutal® (pentobarbitone sodium, 60 mg/ml; Boehringer Ingelheim Pty Ltd, Artarmon, Australia) was used for this
stage. This drug was injected intramuscularly at an approximate dosage of 20 mg/ml per 100 g of body weight.

Several anaesthetic agents have previously been trialled for the experimental protocol used in this study, and the Hypnorm®/Hypnovel® combination has been found to provide the safest and most predictable results, according to Dreyer.\textsuperscript{95} However, Nembutal® was adequate for animal sacrifice and tissue harvesting, although it had a greatly slower onset compared with Hypnorm®/Hypnovel®.

8.3 Thermal insult
Following Hypnorm®/Hypnovel® anaesthesia, individual rats were placed on their back on a specially constructed holding board. Elastic bands were used to prop the mouth open via metal rings attached to the bands and looped around the upper and lower incisors. The elastic bands were attached to the holding board so as to produce a diametrically opposite pull on the incisors, thus gently stretching the mouth open. The tongue was also positioned into the lower ring to retract it away from the operative field. To fully expose the upper right first molar and protect the soft tissues, a small spatula was used to retract the right cheek. This restraining and retracting protocol allowed a single operator to perform the experimental procedures unassisted.

The upper right first molar was frozen for twenty minutes by the continuous application of pellets of dry ice (CO\textsubscript{2} at -81°C, BOC Gases, Adelaide, Australia) held in tweezers. Large tubular pellets provided by the manufacturer were divided using a sharp chisel to produce smaller pellets which approximated the crown size of the rat upper first molar. To avoid contact with the surrounding gingival soft tissues, care was taken to apply the dry ice only to the occlusal aspect of the teeth. Following the application of cold, the tissues were allowed to thaw slowly. The upper left first molar was left unfrozen and served as a control.
The five animals of each individual group were all treated during the same session, with each rat allowed to recover in a separate cage to that containing the untreated members of the group. The animals were wrapped in paper towelling to maintain body temperature during recovery from the anaesthetic agent. All of the rats were monitored by observation following anaesthesia and were again checked several hours after completion of the procedures to ensure full recovery had taken place.

8.4 Sacrifice
The six groups of five animals each were sacrificed via cardiac perfusion fixation at 7, 10, 14, 18, 21, 28 days respectively after the application of the dry ice.

8.5 Perfusion and fixation
Cardiac perfusion was performed in order to adequately fix the jaws. As previously described, the animals were anaesthetized with Nembutal® and the
onset of adequate anaesthesia checked via the absence of reflexes. Each animal was placed on its back in a dissecting tray and the skin over the medial surface of the right hind limb reflected. The femoral vein was exposed by blunt dissection and an injection of heparin B.P. (David Bull Laboratories, Mulgrave, Australia) was administered intravenously at a dose of 0.02ml/100g of body weight (Appendix 12.1.1).

After the administration of heparin, a long midline incision was made from neck to abdomen. Additional relieving incisions were made into the axillary area and the skin and superficial fascia retracted to expose the underlying thoracic and abdominal muscles. The thoracic cavity was entered by sharp scissor dissection at the caudal end of the sternum and at the level of the diaphragm. The rib cage was sectioned in the midline and the two halves reflected outwards to expose the beating heart. The fixative, 4% paraformaldehyde (Appendix 12.1.4), was slowly injected into the left ventricle of the heart and immediately the superior and inferior vena cavae were cut so that there was no venous return to the heart. On completion of perfusion, the maxilla and mandible were dissected out and immersed in the same fixative for 24 hours. After fixation, the specimens were stored in phosphate buffered saline (PBS) at pH 7.4 (Appendix 12.1.3).

8.6 Decalcification

One specimen from each experimental group was radiographed using a standard dental intra-oral x-ray machine at 60KV with an exposure time of 0.1 second. This served as a baseline to assess the progress of decalcification, which was carried out by immersing all of the specimens in 4% EDTA in phosphate buffer at pH 7 (Appendix 12.1.5), with the solution changed twice per week. Further radiographs were taken at regular intervals to monitor decalcification. The specimens were removed from the EDTA solution when there was no longer any visible evidence of mineralised tissue on the radiographs. At this point the specimens were returned to the PBS solution.
8.7 Tissue processing
Specimens were placed in 70% alcohol overnight prior to processing in a Shandon Citadel 2000 automatic tissue processor (Shandon Industries, Pittsburgh, Pennsylvania). Tissue dehydration was carried out in a graded alcohol series prior to embedding in paraffin wax using a Reichert-Jung Histostat. The specimens were trimmed of excess tissue after processing but before embedding, with tissues trimmed away as close to the mesial surfaces of the upper molars as possible. The tissue specimens were carefully oriented during embedding to allow coronal sections to be cut, so that each section would include both the experimental and control teeth (Appendix 12.2).

8.8 Sectioning
Paraffin blocks were mounted in a Leitz 1512 Microtome and ribbons of 7µm sections were cut. Sections were floated onto a water bath thermostatically set at 37°C and lifted onto labelled aminopropyltriethoxysilane (APT) coated slides (Appendix 12.3), with five sections mounted on each slide. The slides were allowed to dry in an oven set at 37°C. The teeth were sectioned to provide serial sections through the furcation region and arranged so that consecutive sections were placed on consecutive slides. Each slide thus carried every tenth section in the series, as illustrated below, for a total of 30 slides per furcation (ie three sets of 10 slides):
In this way, 150 sections were taken from the furcation region of each first molar tooth. The furcation region was chosen as the area of periodontal ligament study as ankylosis was mainly seen in this area in previous studies \(^{76, 95}\). Additionally, 20 sections each were taken from both the mesial and distal root areas of the first molar tooth (ie four slides for each root). These sections were mounted five per slide in the same way as the sections taken from the furcation region; however, in this case every twentieth section was taken. These sections were used for trial runs while developing the various staining protocols and to serve as negative controls for the immunostaining.

### 8.9 Haematoxylin and Eosin Staining

To gain a general overview of the tissues of interest, three slides per animal were stained with Mayer Lillie Haematoxylin and Eosin (H&E) (Appendix 12.4). For this purpose, the first, eleventh and twenty-first slides from each furcation series were used (ie the first slide from each set of ten slides). These slides were used to determine the presence of ankylosis and root resorption within the furcation areas of both the control and experimental teeth.
8.10 Immunostaining

Several trial staining runs were conducted to determine the exact protocol for optimum immunostaining with both cytokeratin AE1/AE3 (Cell Marque, Hot Springs, AR, USA) and PGP 9.5 (Ultraclone, Rossiter's Farmhouse, Wellow, Isle of Wight, UK). The first problem encountered was antigen retrieval, as it was found that the tissues of interest were very fragile. Initially antigen retrieval was attempted by boiling the mounted sections in citrate buffer, but this method caused most of the tissue to lift off the slides, thus destroying the area of interest. A second run was carried out using citrate buffer heated to 85 degrees, but this did not significantly improve the preservation of tissue architecture. Two further test runs were performed, one using enzyme digestion as the method of antigen retrieval, the other carried out without any active antigen retrieval procedure. Both of these methods allowed successful immunostaining without disruption of the tissue, although significant background staining was noted when the antigen retrieval step was omitted. On the basis of these findings, it was decided to use enzymatic antigen retrieval, and this was performed using a 0.5mg/ml solution of Trypsin II in PBS at 37 degrees for 3 minutes.

The second aspect to be determined was the optimum dilution of the primary antibody. Previously reported dilutions for PGP 9.5 have ranged from 1:250 to 1:8000. In this study, a dilution of 1:20,000 was used. This dilution was suggested by the immunocytochemistry laboratory at the Institute of Medical and Veterinary Science (IMVS) which uses this antibody on a regular basis, and it was found to produce good staining results with the tissue under investigation in this study. For the cytokeratin AE1/AE3, an initial trial run was conducted using a dilution of 1:2000, which had been employed successfully in a previous study within this department. When this did not produce an adequate staining, another staining run was carried out using dilutions of 1:100, 1:500 and 1:1000. Both 1:100 and 1:500 produced good staining reactions, and the dilution of 1:500 was chosen for the main experiment.
A multi-species immunostaining detection kit (Signet Laboratories Inc, Dedham, Massachusetts) was used during the immunostaining protocols for blocking serum, secondary antibodies, streptavidin peroxidase, and staining with 3, 3'-diamino benzidine (DAB). This system worked well when using the cytokeratin antibody marker, but significant background staining was noted when the PGP 9.5 antibody was used. Background staining was reduced when 3% normal horse serum was used in place of the blocking serum from the detection kit; however, the best results were obtained when the detection kit was not used at all and the secondary antibody and streptavidin peroxidase were made up fresh at the time of staining. Two thirds of the slides stained with PGP 9.5 were prepared in this way using the facilities of the immunocytochemistry laboratory at the IMVS (Appendix 12.5).

8.10.1 Cytokeratin AE1/AE3
This antibody was used to identify epithelial cells within the periodontal ligament. An internal positive control, the epithelial lining of the palatal mucosa, was used to assess the success of each staining run. In addition, a separate positive control slide was also employed, along with a negative control slide, to monitor the staining process. The positive control was human buccal mucosa obtained from a biopsy sample of fibroepithelial hyperplasia. The negative control consisted of one slide of sections from the mesial or distal root areas, as outlined above, which was treated in the same way as the experimental slides except that the primary antibody was omitted and replaced with PBS at the appropriate stage. The slides used for staining with the cytokeratin marker were the next slides in the series after those used for H&E staining (ie the second slide from each set of ten slides).

8.10.2 PGP 9.5
This antibody was used to identify nerve fibres within the periodontal ligament. Positive and negative controls were used as per the cytokeratin stain, with the
internal positive control being the major nerve bundles within the mucosa on either side of the hard palate. Staining with PGP 9.5 was carried out on the third slide from each set of ten slides. In this way, successive sections were stained with H&E, cytokeratin and PGP 9.5, allowing easy comparison.

8.11 Cell counts
Cell counts were undertaken for both the epithelial cells and nerve fibres within the periodontal ligament in the furcation region of the first molar teeth on both the experimental and control sides.

8.11.1 Epithelial cell counts
All of the sections stained with cytokeratin AE1/AE3 were used for epithelial cell counting. The periodontal ligament of the furcation region was divided into equal buccal, middle and palatal thirds using a grid with 300 x 300µm squares when viewed at 10X magnification and all epithelial cells which were visible in each square at this magnification were recorded. Each distinct single cell or consolidated group of cells was counted as one, regardless of the number of individual cells in each group, due to the difficulty in discerning the exact number of cells in each cluster. Counts were made for both the control and experimental teeth in each section. Counts were not made for teeth in which the furcation was not clearly visible in a particular section, or where the section was damaged in the furcation region. Scoring of the epithelial cell counts was entered into a spreadsheet format using Microsoft Excel 2003.

8.11.2 Nerve fibre counts
Due to the fact that it was difficult to identify the nerve fibres, especially the fine endings, counts were made at 40X magnification and every fifth section stained with PGP 9.5 was used for the recordings. Each furcation was first viewed at 10X magnification and divided into thirds in the same manner as for the epithelial cell
counts. The actual counting was undertaken at 40X magnification, with each individual fibre recorded. In addition to the actual number of nerve fibres observed in each area of the furcation (ie buccal, middle or palatal), the relationships of the fibres were also recorded. The nerve fibres were classified firstly on the basis of whether they were associated with blood vessels or not, then classified with regard to their location within the periodontal ligament. This latter classification included recording whether the fibres were located in the alveolar bone, middle or root thirds of the ligament, and whether they were associated with areas of resorption or ankylosis. As with the epithelial cell counts, both the experimental and control teeth were counted in each section. A Microsoft Excel spreadsheet was set up to record the nerve cell counts.

8.12 Resorption and ankylosis

During the counting procedure for the epithelial cells, the presence, amount and location of any resorption and ankylosis was also recorded. The 300 x 300µm grid at 10X magnification was used again to allow localisation of each area of resorption or ankylosis as well as an approximation of the amount of root surface affected by either condition. These recordings were made for both the experimental and control teeth, using the same sections which were employed for the epithelial cell counts, and were entered into separate sheets in the same spreadsheet used for the epithelial cell counts.

8.13 Microscopic imaging

Final viewing and recording of photomicrographs of specimens was carried out using the facilities of the Adelaide Microscopy Centre. Sections were viewed using an Olympus BX51 microscope with attached digital camera, and the areas of interest were examined using 10X, 20X and 40X objective lenses. Image capture was carried out with AnalySIS LS Research imaging software using a personal computer connected to the microscope camera. Adobe Photoshop CS Version 8.0 was used for adding labels and markers to the images to be included as figures within the text.
8.14 Statistical analysis

Statistical analysis of the data was carried out using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). The data for the epithelial cell and nerve counts were first plotted on histograms to determine whether the data were normally distributed. To assess for changes in epithelial cell count according to several predictor variables, Poisson GEE regression models were fitted to the data. Univariate analyses were carried out first, followed by a multivariate analysis, in which the influence of each variable on epithelial cell count was interpreted as its relationship after adjusting for all other variables included in the model. The variables investigated were time period, location within the periodontal ligament (ie buccal, middle or palatal third), side (ie experimental or control tooth) and presence or absence of ankylosis. The influence of the epithelial cell count on the probability of ankylosis was assessed using a log binomial GEE regression model. This model was used due to the fact that the outcome (ankylosis) was treated as a binary variable rather than a count variable.

The relationship between resorption and several variables was also investigated using a log binomial GEE regression model. For the purpose of this analysis, resorption was treated as a binary yes/no variable. Univariate analysis was carried out first, followed by a multivariate model. The variables included in the models were time period, location within the periodontal ligament, side, and epithelial cell count.

Nerve counts were assessed relative to a number of variables using Poisson GEE regression models in a similar manner as for epithelial cell counts. The variables included in the models were time period, location within the periodontal ligament, and side. Location within the periodontal ligament was considered both in relation to the tooth axes (ie buccal, middle and palatal), as well as in relation to the width of the periodontal ligament (ie close to alveolar bone, close to the
tooth root surface, or centrally located within the ligament). Once again, univariate analyses were carried out first, followed by multivariate modelling.

Finally, the existence of any relationship between nerve and epithelial cell counts was investigated. A Poisson GEE regression model was used, after adjusting for the variables of side and location within the periodontal ligament.

8.14.1 Method error
To assess the reliability of the counts, recounts were undertaken for epithelial cells and nerve fibres on 37 and 47 randomly selected sections respectively. The degree of agreement between the two sets of data was assessed with a weighted kappa statistic. The data were categorised into four groups to facilitate the use of this analysis, based on the number or cells or fibres counted. The categories used were (count of) 0, 1, 2 and 3 or more.