AN AUTORADIOGRAPHIC AND DEVELOPMENTAL STUDY OF TRANSALVEOLAR FIBRES IN THE MOUSE MANDIBLE

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

The Department of Dental Health
The University of Adelaide
December 1978

Awarded: April 1979

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SUMMARY

The presence of transalveolar tooth to tooth fibres and anatomical descriptions of their arrangement in the alveolus of the mouse, are reported in the recent dental literature (Cohn, 1970, 1972a; Dunstan, 1975). It is hypothesised that these fibres play a functional role in tooth support and in the stability of intramaxillary tooth relations (Melcher and Walker, 1976; Orban, 1976).

Discordant anatomical descriptions of transalveolar fibres are given by Cohn (1972a) and Dunstan (1975). Furthermore, the maintenance of the transalveolar fibre system during physiologic tooth movements is not considered by these workers. The present investigator found that the portions of transalveolar fibres in bone are described by previous workers using different terminology (Stein and Weinmann, 1925; Enlow, 1968). These investigators report the formation of patterns of Sharpey fibres in bone due to physiologic tooth movements.

The results of this investigation showed that there was no remodelling of transalveolar or Sharpey fibres in bone, whereas evidence of rapid remodelling was observed in principal periodontal fibres. During physiologic tooth migration, Sharpey fibres were shown to be incorporated into depository socket surfaces, forming patterns which were maintained in the bone until it was resorbed. Developmentally, transalveolar fibres were not formed by incorporation of tooth to tooth fibres from the supracrestal tissues into the bone matrix. Each tooth formed its own Sharpey fibre insertions independently. No selective reattachment of periodontal fibres to

Sharpey fibre remnants at resorptive surfaces was observed. Furthermore, no mechanism was found for the remodelling of the intraosseous portions of the proposed transalveolar fibres during physiologic tooth movements.

Various patterns of Sharpey fibres in bone were seen depending on the stage of eruption of the teeth and the plane of tissue sectioning. However, a distinct tooth to tooth fibre system perforating alveolar bone was not confirmed by the study. It was concluded that Sharpey fibre patterns, formed in bone due to tooth drift, have previously been incorrectly interpreted as the intraosseous portions of tooth to tooth fibres.

The structural significance of Sharpey fibres in alveolar bone and the possible relation of their orientation to force dissipation in the masticatory system, was suggested as an interesting area for further research. It was considered, however, that Sharpey fibres incorporated into bone during physiologic drift and reflecting the previous directions of such movements, were unlikely to have the same role as the rapidly turning over periodontal fibres.

SIGNED STATEMENT

To the best of my belief this report contains no material which has been accepted for the award of any other degree or diploma in any university. Furthermore it contains no material previously published or written by another person except where due reference is made in the text.

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ACKNOWLEDGEMENTS

I gratefully acknowledge the assistance of my supervisor Dr M.R. Sims, Reader in Orthodontics, in the preparation of this report. I also wish to thank Mrs L. McMahon for her technical advice with histologic techniques and Mr D.E. Smale who has freely made available the facilities of the Oral Pathology Laboratory whenever requested. I am indebted to Professor A.W. Rogers of the Flinders University Medical School, for his valuable assistance in demonstrating the technique of autoradiography used in this project. I also wish to thank Mrs M. Cummings for her excellent work in typing the manuscript.

CHAPTER 1

INTRODUCTION

The conventional understanding of periodontal anatomy is challenged by Cohn (1970, 1972a) who reports that the molar teeth of mice are interconnected by collagen fibre bundles passing through the alveolar bone. He confirms that the principal periodontal fibres are arranged into alveolar crest, horizontal, oblique and apical groups as is described in the dental literature (Ten Cate, 1969; Goldman and Cohen, 1973; Orban, 1976). Cohn (1972a) and Dunstan (1975) report that each of these fibre groups in the mouse is continuous with the corresponding fibre groups of the adjacent periodontal ligament, through the alveolar bone. However, anatomical descriptions given by each of these authors of the proposed transalveolar fibre system vary significantly. Further experimental evidence confirming the presence of transalveolar fibres in other species has been published (Quigley, 1970, Cohn, 1972b, 1973, 1974, 1975; Edwards, 1975).

The reported presence of another tooth to tooth fibre system, in addition to the previously described and demonstrated transeptal fibre system, has introduced new areas requiring research. In the present investigation, radioactive proline was selected to evaluate the rate of turnover of transalveolar fibres in the mouse mandibular molar segment. Routine histologic techniques were also used to study the development of transalveolar fibres and their maintenance as the teeth drift physiologically throughout life.

The tendency of orthodontically moved teeth to return towards pretreatment positions is widely reported and not yet fully explained. Reidel (1969) reviews the changing concepts of the aetiology of relapse. Factors implicated in relapse are broadly grouped into those which are localized around the tooth roots and those which exert their effect indirectly by pressures on the crowns.

With regard to relapse potential, two classes of collagen fibres associated with the tooth root are recognised. Immediately following orthodontic tooth movement, bone to tooth periodontal fibres appear stretched (Reitan, 1959). However, when the teeth are retained in their new positions, the fibres remodel and resume the normal orientation of the periodontal ligament. Consequently, they are not considered as a major factor in relapse. The supracrestal soft tissues, transeptal and gingival fibres, do not undergo the same rapid remodelling, however, and maintain their stretched appearance even after long periods of retention. Edwards (1968), Boese (1969) and Brain (1969) report increased stability of orthodontic tooth movements following excision or transection of the supracrestal soft tissues. Erikson, Kaplan and Aisenburg (1945) also suggest that transeptal fibres may be implicated in relapse following orthodontic treatment.

Moss and Picton (1973) report that excision of supracrestal soft tissues, following interproximal tooth reduction, reduces the rate of approximal drift in monkey molar segments. Drift is not eliminated, however, and these workers are unable to satisfactorily account for the remaining movement. Similarly, excision of the supracrestal tissues following experimental rotation only decreases the tendency

to relapse (Edwards, 1968). It is suggested that the transalveolar fibre system may be implicated in orthodontic relapse (Quigley, 1970; Cohn, 1972a). A time scale of transalveolar fibre turnover would provide significant new information related to the problem of retention following orthodontic tooth movement.

CHAPTER 2

AIMS OF THE INVESTIGATION

This study aimed to investigate two aspects of the transalveolar fibre system in order to test the reported hypotheses that they play a functional role in both tooth support and the stability of intramaxillary tooth relations.

Firstly, the rate of remodelling of transalveolar fibres in the alveolar bone of the mouse was to be examined using autoradiography to estimate turnover time.

Secondly, the development of transalveolar fibres was to be described from infancy to adulthood. An explanation of the differences between the anatomical description of transalveolar fibres given by Cohn (1972a) and Dunstan (1975), was also sought.

As a result of these investigations it was anticipated that the functional significance of transalveolar fibres might be clarified and further areas for research related to their function be suggested.

CHAPTER 3

REVIEW OF THE LITERATURE

SHARPEY FIBRES

The name Sharpey fibre dates from the latter half of the 19th Century (Kolliker, 1860; Muller, 1860; cited by Jaffe, 1972, p.50). It is used to describe the collagen fibres, which were first identified microscopically by these workers, inserting into the roughened bone surfaces at areas of soft tissue attachment. Reference to Sharpey fibres and the investing bundle bone is made in most histological texts since. Stein and Weinmann (1925) describe bundle bone, hereinafter termed Sharpey fibre bone, around the teeth and refer to the insertion of Sharpey fibres as a mechanism of attachment of the teeth to the jaws. Principal periodontal fibres incorporated into cementum are also termed Sharpey fibres (Orban, 1976).

Cohn (1970, 1972a) reports tracing Sharpey fibres, in serial sections of the alveolar bone of mice, across the entire thickness of the interdental and interradicular septa and states that they are continuous with the periodontal fibres from the adjacent tooth roots. On the basis of this unique anatomical description, he hypothesises that Sharpey fibres may have functional significance in addition to that of attachment of the teeth to the jaws. He introduces the name 'transalveolar' fibre to embody his concept of a functional tooth to tooth fibre which perforates alveolar bone. Quigley (1970) provides

support to the concept by confirming that Sharpey fibres in the mouse mandible pass without interruption across the entire width of the interseptal bone.

ANATOMY OF TRANSALVEOLAR FIBRES

Cohn (1972a) presents a detailed anatomical description of transalveolar fibres in the mouse. Cohn (1972b) also reports a similar pattern in the marmoset. Histologic examination of the mandibles of 20 adult albino mice cut at 8 microns and stained with Van Geison stain or colloidal iron form the basis for his findings (Cohn, 1972a). Serial sections were cut buccolingually and mesiodistally in the long axis of the teeth. Cohn (1972a) states that individual fibres can be traced from the cementum of one tooth, across the periodontal ligament and through the alveolar bone to either the adjacent tooth root or the periosteum. From his work, he develops a schematic representation of the transalveolar fibre system (Fig. 1).

The reported arrangement of fibres within the periodontal ligament is in agreement with observations of Zwarych and Quigley (1965), with more, somewhat finer fibres embedding into cementum and fewer, somewhat thicker fibres embedding into alveolar bone. Between these attached Sharpey fibres the main bundles branch and interweave in a three dimensional network of finer fibres. Despite the interchange between bundles, it is claimed that each fibre maintains continuity across the periodontal ligament.

Periodontal anatomy is usually described in terms of the alveolar crest, horizontal, oblique, and apical tooth to bone fibre groups

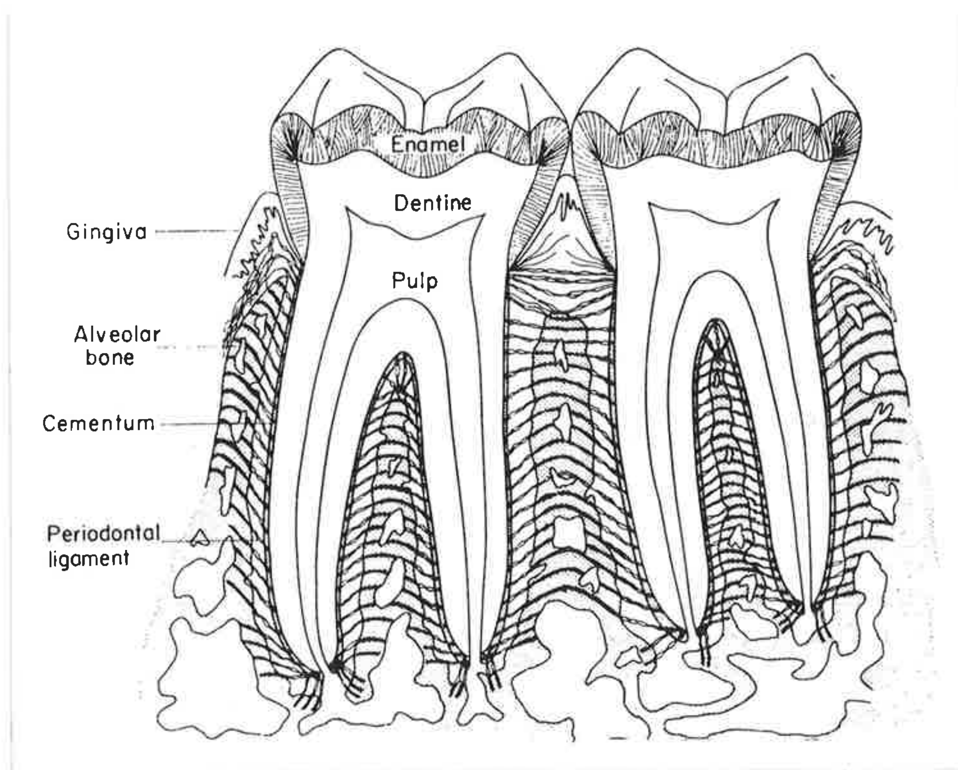

Fig. 1

Schematic representation of transalveolar fibres in the mouse mandibular molar segment in the mesiodistal plane as depicted by Cohn (1972a).

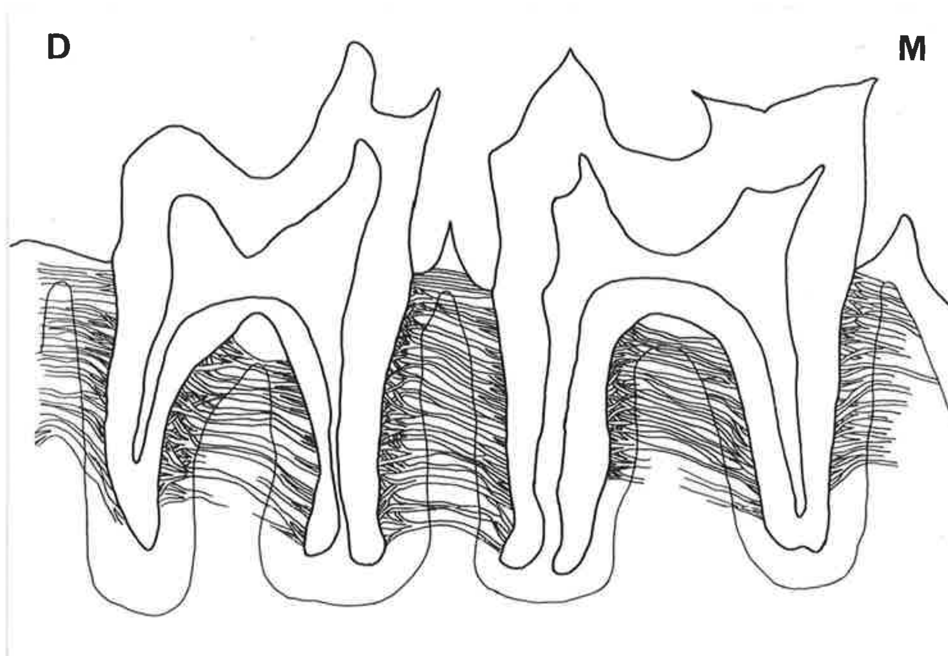

Fig. 2

Schematic representation of transalveolar fibres in the mouse from Dunstan (1975). Dunstan does not show the V-shaped junctions of alveolar crest fibres and illustrates an assymetrical fibre pattern with transalveolar fibres directed superiorly and distally from the mesial side of the septal bone. D, distal; M, mesial.

(Orban, 1976). Cohn (1972a) claims that corresponding groups of periodontal fibres from adjacent tooth roots are continuous through the alveolar bone via Sharpey fibres.

Cohn's description of the anatomy of perforating Sharpey fibres (transalveolar fibres) is similar for each of the three molar teeth. In the interdental region, periodontal fibres of the alveolar crest group descend from the cementum of each tooth and form a V-shaped pattern in the alveolar crest. On the buccal and lingual sides and the mesial of the first molar tooth, the alveolar crest fibres penetrate the crestal bone and emerge to mingle with fibres of the periosteum.

The small horizontal group of periodontal fibres is continuous with the corresponding group from the adjacent tooth, the overlying periosteum or gingival connective tissue. The oblique group of fibres pass superiorly from the cementum, and enter the bone. They then arc apically and become continuous with fibres from the adjacent tooth (Fig. 1). Cohn (1972a) states that their course in the bone is sinuous, although the great amount of interweaving of the periodontal ligament fibres is not evident. Those fibres on the vestibular surfaces, and on the mesial of the first molar, arc similarly and become continuous with fibres of the periosteum. The apical fibres descend into the bone and apparently lose their identity at an unspecified depth by uniting with intrinsic bone matrix fibres.

Cohn (1975) states that not all Sharpey fibres perforate the alveolus. There are significant differences in the interpretations of results, over and beyond species differences, between the reports of

studies on man and mouse. In the latter, Cohn's (1972a) schematic diagram indicates that cancellous spaces are due to secondary remodelling of Sharpey fibre bone (Fig. 1). In the report on his findings in man, Cohn (1975) describes Sharpey fibres penetrating only lamellar bone lining the tooth socket and not the cancellous bone, suggesting that fibres on each side of such cancellous spaces may arise from each periodontal ligament. Furthermore, in his 1975 article Cohn no longer describes arcing of the transalveolar fibres as a smooth curve from one set of oblique fibres to the other, but an angled V-shaped union at a reversal line in the Sharpey fibre bone where the fibres from each tooth join.

Dunstan (1975) supports the concept of a tooth to tooth fibre system and suggests, therefore, that teeth should be considered as groups rather than as individual units. He reports observations of 8 micron serial sections of adult mouse mandibular molar segments cut in the mesiodistal plane. His results vary from those of Cohn in the anatomical descriptions of the transalveolar fibres. Dunstan claims that arcing of transalveolar fibres is not commonly seen and that they maintain a fairly straight course in bone (Fig. 2). Furthermore, the V-shaped patterns of alveolar crest fibres in bone are not illustrated in his report. He shows transalveolar fibre patterns to be assymetrical, with the portion of the transalveolar fibres in bone directed superiorly and distally from the mesial side of the septa (Fig. 2).

Bernick *et al.* (1974) fail to confirm the presence of transalveolar fibres in their histological study of marmosets. Baron (1973a) reports no evidence of a tooth to tooth fibre system in mice. He traces Sharpey

fibres in serial sections and finds that they always terminate at a reversal line in bone, or at a resorptive bone surface.

DEVELOPMENT OF TRANSALVEOLAR FIBRES (PERFORATING SHARPEY FIBRES)

The development of transalveolar fibres is not discussed in the literature, although Edwards (1975), comments that they may arise by incorporation of transeptal fibres into the alveolar crest as it develops. The formation of Sharpey fibres is described briefly in the normal sequence of development of mouse molars and their surrounding structures (Cohn, 1957; Atkinson, 1972). The prenatal and postnatal morphogenesis of mouse molars is described by Mahn (1890), Gaunt (1956) and Hay (1961). However, these workers do not describe the development of the periodontium.

The gomphosis (tooth, periodontal ligament, bone, attachment apparatus) is a particularly stable and successful evolutionary step which first appeared approximately 200 million years ago. It was rapidly incorporated into the mammals which were evolving from their reptilian predecessors at that time (Noble, 1969). Picton (1976) states that a major advantage of the gomphosis is that it allows for compensatory movements due to tooth wear. Despite the great variation in size and position of the tooth socket in different species, there is a strong similarity in its form (Noble, 1969).

Ainamo and Talari (1976) recognise three forms of the gomphosis in mammals associated with:

1. Continuously growing teeth

2. Continuously extruding teeth

3. Teeth which erupt continuously with their investing tissues without further growth of the tooth except by deposition of cellular cementum.

The molar teeth of mice and several other animals which will be referred to herein fall within the third category.

EMBRYOLOGY OF THE DENTAL STRUCTURES

The origin of the enamel and enamel-forming structures of the teeth is from surface ectoderm (Ten Cate 1969). At an early stage *in utero*, prior to neural fold fusion, cells of the neural crest migrate as an intact layer between the ectoderm and the underlying mesoderm (Johnston and Listgarten, 1972; Noden, 1973). These cells are indistinguishable from other mesoderm cells unless marked by autoradiography or localized histochemically by their high glycogen content. They are termed either neural crest or ectomesenchymal cells.

During their migration, neural crest cells appear to receive inductive stimuli from various types of tissue. Depending on the nature of these stimuli, the crest cells may cease to migrate and differentiate into various types of skeletal and connective tissue, as well as neural and other tissues, (Johnston and Listgarten, 1972). These workers claim that odontoblasts are of neural crest origin.

The nature of interaction between the ectomesenchyme and ectoderm has been investigated. The basement membrane appears to act as a selective filter through which various products pass. These products are essential for subsequent inductive changes (Slavkin, 1972, 1974).

Kollar (1972) shows by selective cell transplantations that the inductive potential for dental structures lies within the ectomesenchyme, although he emphasizes the importance of the basement membrane of the ectoderm in polarizing cell populations.

Development of the tooth primordium proceeds with downward proliferation of the ectoderm into the ectomesenchyme. By the time the bud of ectoderm invaginates into the early cap stage, the entire ectodermal mass is surrounded by cells which, on the basis of histochemical glycogen identification are ectomesenchyme (Ten Cate, 1969; 1972). Thus the dental papilla which gives rise to the odontoblasts, dentine, and an investing layer around the tooth primordium are considered to be of ectomesenchymal origin. The investing layer is a definitive fibro-cellular layer surrounding the tooth primordium and its capsule-like nature allows a developing tooth to be isolated from the jaws with minimal loose attached tissue. Ten Cate and his co-workers in numerous transplantation studies endeavour to clarify the structures which the ectoderm and ectomesenchyme of these tooth germs finally form.

Ten Cate, Mills and Solomon (1971) report an experiment in which they dissect out 181 mouse molar tooth germs, label these with ^{3}H thymidine and replant them subcutaneously in mice of the same strain. continuing development of the crown, followed by formation of the root, cementum, periodontal ligament and 'alveolar' bone, is reported in 43% of the teeth. Tracing the radioactive label clearly shows that the cementoblasts and some periodontal ligament fibroblasts are derived from the implanted tooth germs. However, due to dilution of the label

in many divisions, it is not clear if the surrounding bone or if all fibroblasts are of donor origin. Freeman and Ten Cate (1971) suggest a perivascular mesodermal source of some fibroblasts in the developing periodontium. The possibility of fibroblasts arising from two sources is thereby raised.

Ten Cate, Mills and Solomon (1971) note that there is an infiltration of lymphocytes around their implanted tooth germs, and significantly, external to the 'alveolar' bone. They suggest that this is evidence of a rejection reaction, indicating that cementum, periodontal ligament and alveolar bone arise from donor ectomesenchymal tissue.

Ten Cate and Mills (1972) follow up this observation with an ultrastructural examination of the implants and conclude that a rejection reaction does occur external to the 'alveolar' bone, thus supporting their previous hypothesis that bone, ligament and cementum are derived from the ectomesenchymal investing layer.

Main (1966) cultures mouse tooth germs on gelatin sponges for periods up to 37 days. After this time, all semblance of tooth germ morphology is lost and the culture consists of only a few scattered ectodermal and ectomesenchymal cells. When these cells are harvested and implanted subcutaneously into adult mice, they resume their developmental potential and form tooth, periodontal ligament and bone (Ten Cate and Mills, 1972).

Barton and Keenan (1967) suggest from an examination of subcutaneously transplanted tooth germs that on a morphological basis

the entire periodontium, including alveolar bone, is of donor origin. Further experimental support for the hypothesis is found in the reports of Atkinson and Lavelle (1970) and Freeman, Ten Cate and Dickinson (1975). In the former study Atkinson and Lavelle (1970) implant tooth germs into the femurs of rats, an area which normally repairs defects with bone. They observe that the teeth continue to develop and form a gomphosis. In the latter work, Freeman *et al.* (1975) implant tooth germs from one day old mice into the parietal bone of their respective mothers. Defects in the parietal bone are known to normally repair with fibrous tissue. However, these workers find that the implanted tooth germs develop and form new bone as well as periodontal ligament. They consider the new bone to be derived from the implanted tooth germs. Furthermore, Ten Cate (1975) shows that bone is deposited coincidentally with periodontal ligament formation in the mouse and therefore suggests that the tooth germs generate their own periodontal ligament and alveolar bone.

Alternatively it is suggested that the observation of development of periodontal ligament and bone around tooth germ implants may reflect an inductive potential of the ectodermal structures (Hoffman, 1960). This worker considers that only ectodermal structures are transplanted with tooth germs. Although Ten Cate *et al.* (1971) consider it most likely that Hoffman (1960) did transplant tooth germs with their ectomesenchymal investing layer, it is not yet proven that the entire periodontium is of ectomesenchymal origin. The possibility exists that the ectodermal tissue exerts an inductive influence on other mesoderm of the jaws.

DEVELOPMENT OF THE TEETH AND PERIODONTIUM

Currently it is thought that the investing layer is responsible for the development of cementum, periodontal ligament and alveolar bone (Ten Cate 1976). This layer grows with the developing tooth, becoming elongated as the roots form (Ten Cate, 1969). On its inner aspect is a layer of loose connective tissue in contact with the root. On its outer surface is a layer of loose connective tissue in contact with bone. The appearance of three zones in the developing periodontal ligament is described in primary teeth and in teeth which form without primary predecessors but not in succedaneous teeth (Furstman and Bernick, 1972). Orban (1972), describes this histologic appearance as an intermediate plexus. The middle fibrous layer becomes obliquely orientated and thicker as the tooth develops. The maturation of tissue commences at the cemento-enamal junction and continues apically as the root elongates. At the cemento-enamel junction there is a change in the orientation of the fibres and elongated cells of the investing layer as they sweep over the crown.

Cohn (1957) and Orban (1976) describe the process of odontoblast induction by Hertwig's epithelial root sheath. Ten Cate (1969) details how the cervical parts of the root sheath become slightly separated from the newly deposited dentine, and fibroblasts in the loose connective tissue between the root and the middle fibrous layer elaborate collagen fibrils which become positioned between the root and epithelial cells. As development proceeds, the epithelial remnants move out into the connective tissue and cementoblasts mineralize the first layer of cementum which has a characteristic lack of organised Sharpey fibres

(Selvig, 1964, 1965; Ten Cate, 1969). Further cervically, collagen fibres progressively gain attachment to the cementum. These fibres become continuous with the fibres of the investing layer which run in a superior oblique direction (Eccles, 1959). Cohn (1957) claims that the cementum does not become mineralized until the teeth erupt.

Paynter and Pudy (1958) claim that the first layer of non-Sharpey fibre cementum may be of epithelial origin derived from Hertwig's epithelial root sheath. In a more recent publication Stahl and Slavkin (1972) also emphasize the importance of epithelial mesenchymal reactions for inducing cementoblasts. They claim that the first layer of cementum may be of epithelial origin and that outer cementum is a subsequent mineralization of Sharpey fibres. This mechanism would provide the simplest solution to the problem of establishing an intact dentino-cemental junction.

From the outer zone of the investing layer, fibres and elongated cells become aligned in a superior oblique direction and attach to the bone completing the formation of the oblique fibre group. Mice have a monophyodont dentition, the first molars erupting at approximately 16 days (Cohn, 1957; Atkinson 1972). Ten Cate (1975) shows that the orientation of periodontal fibres in relation to the first molars of mice commences between 11 and 13 days. Jones and Boyde (1974) show that prior to the development of Sharpey fibres at the bony margin, the intrinsic bone matrix fibres encircle the tooth horizontally. After Sharpey fibres become attached, a change in the pattern occurs as intrinsic fibres become ordered around the Sharpey fibres. When the initial periodontal fibre attachment is formed, small fibres are

seen emanating from the bone in an inferior oblique direction towards the root (Grant and Bernick, 1972). As the teeth erupt, a change of orientation of the crestal fibres occurs forming the classically arranged alveolar crest, horizontal and oblique fibre bundles of the periodontal ligament (Ten Cate, 1969).

Eccles (1959) and Trott (1962) consider that functional forces transmitted by the gum pads are responsible for further periodontal fibre development. Bernick (1960) claims that the periodontal fibres become thicker with function. Atkinson (1972), however, found no relation between the developmental changes in the periodontium and stages of eruption of the teeth. Barton and Keenan (1967) show that Sharpey fibres form in non-functional, subcutaneously transplanted tooth germs.

In scanning electron microscope studies of developing teeth from monkeys and rats, Jones and Boyde (1974) show that the diameter of collagen fibre bundles is remarkably consistent both pre and post eruption, and interestingly, between species. They suggest that the size and function of osteoblasts may be important in determining the maximum permissible size of Sharpey fibres.

As the teeth come into occlusion the transeptal fibres form as fibres from each tooth anastomose and join (Cohn, 1957; Eccles, 1959; Ten Cate, 1969; Furstman and Bernick, 1972; Levy *et al.*, 1972; Orban, 1976). Ten Cate (1969) states that it is the fibres of the investing layer over the crown prior to eruption that are rearranged to form the transeptal ligament and the free gingival fibres as the teeth erupt.

FUNCTIONAL CONSIDERATIONS OF TRANSALVEOLAR FIBRES

Dunstan (1975) makes an interesting observation on the difference in fibre bundle patterns in the periodontal ligament on the mesial and distal of mouse molars. He states that the fibre bundles on the distal facing alveolar surface are consistently thicker than those on the mesial. He suggests that a functional distal force vector may cause the observed distal physiologic drift of the mouse mandibular molars. Consequently, the fibre bundles attaching the mesial surfaces of the roots to the distal surfaces of the alveoli are thicker. Dunstan (1975) also reports the path of Sharpey fibres within bone to be generally in line with the major fibre bundles of the periodontal ligament and states that this reflects the functional demands of mastication. Rodbard (1970) claims that the amount and orientation of collagen in tissue is determined by the vector of tensional stress.

Cohn (1972a) considers that transalveolar fibres may be involved in the positioning of teeth, relative to other teeth and within the alveolus. He thereby raises the possibility of these fibres being involved in relapse following orthodontic tooth movements.

Quigley (1970) discusses the possible function of fibres perforating alveolar bone. He observes that the fibres are spiral in nature. This finding is supported by illustrations in his work as well as in that by Dunstan (1975). He suggests that such an arrangement may allow stretching of fibres within the bone during severe stress and thereby prevent rupture at a hard surface. Boyde (1972) considers that the degree of mineralization of Sharpey fibres represents a functional adaptive mechanism designed to distribute load evenly between the

fibres at a surface. Cohn (1975) and Dunstan (1975) state that the orientation and distribution of transalveolar fibres represent a functional adaptation to occlusal forces.

The degree of calcification of Sharpey fibre bundles is reported by several workers. Frank *et al.* (1958) in an electron microscope examination of undecalcified sections of alveolar bone, find that the Sharpey fibres are generally unmineralized. They also note a woolly appearance of the fibres and suggest that they are enshrouded with ground substance. Baron (1973b) confirms the presence of a partially mineralized sheath of ground substance around Sharpey fibres.

Selvig (1964) considers that cementum Sharpey fibres are fully calcified. Selvig (1965), studying alveolar bone Sharpey fibres, finds that they have unmineralized cores of varying diameters up to 20 microns. In this study he also revises his previous report (Selvig, 1964) by noting occasional uncalcified cores in cemental Sharpey fibres.

Shackleford (1973) using a microradiographic technique in a study of dog alveolar bone finds that many Sharpey fibres contain uncalcified cores. These cores are larger and more uniformly distributed in the alveolar crest region. Other fibres are fully calcified. He also reports that bony protruberances often impinge on uncalcified fibres and suggests that this may inhibit slippage. Combined with fully calcified fibres, resistance of the anchored tooth to functional forces would thus be provided.

Jones and Boyde (1974) use the scanning electron microscope to observe Sharpey fibre bone on socket surfaces and in fractured socket

walls in both organic and anorganic specimens. In their discussion of Sharpey fibres in cementum, alveolar bone and sutures, they state that the degree of calcification varies along the length of the individual Sharpey fibres. Aided by the depth of focus of the scanning electron microscope and the ability to scan topographically uneven surfaces, they found that incremental lines represent deposition of patches of bone over discreet areas. They compare the degree of calcification of Sharpey fibres in areas where incremental lines are well spaced, signifying rapid apposition, to that in areas where the lines are closer, signifying less rapid apposition, and conclude that the degree of calcification in Sharpey fibres is inversely related to the rate of ossification of the investing matrix.

Trott (1962) describes a difference in the reaction to Masson's trichrome stain of the Sharpey fibres within the bone which stain red, compared to their extension in the ligament which stain green. The observation of some collagen fibres selectively taking up acid fuchsin dye is reported by workers in other fields. Craik and McNeil (1966) show that the collagen in the dermis normally stains green with Masson's trichrome stain. However, when the skin is placed under tension, the collagen stains red.

TRANSALVEOLAR FIBRES DURING TOOTH MOVEMENT

The phenomenon of drift of the teeth is first reported by Hunter (1778, cited by Moss and Picton, 1967). Stein and Weimann (1925) observe horizontal sections of human jaw and detect a conspicuous difference between the appearance of the alveolar bone

on the mesial of the teeth and that on the distal. On the mesial they report a thin layer of Sharpey fibre bone whilst on the distal they observe a thick layer of Sharpey fibre bone. They interpret their observations to correlate the pattern of Sharpey fibre bone apposition with the direction of physiologic migration in man, and state that it is towards the midline. On the basis of the distribution of Sharpey fibre bone, these workers assess the direction and rate of tooth migration.

There are no reports in the literature on the remodelling of transalveolar fibres as teeth drift physiologically. However, the incorporation of Sharpey fibres at depository surfaces and the reattachment of periodontal fibres to resorptive surfaces are more widely researched.

Sicher and Weinmann (1944) study histologic evidence of migration of teeth using the rat as an experimental animal. By observing the areas of Sharpey fibre bone apposition and resorption, these workers determine the rate and direction of migration of the rat molars. In their histologic horizontal sections, they report a uniform picture of apposition of bone incorporating principal periodontal collagen fibres on the mesial socket wall. On the distal socket wall they observe three different patterns amongst their specimens. They suggest the three patterns represent consecutive stages in the resorption of bone and the reattachment of periodontal fibres as the root moves distally towards the bone. In the first stage typical osteoclastic resorption severs the periodontal fibre attachment. The resorption extends deeper into the socket wall than

the adjacent level of alveolus. In the second stage, thin layers of bone are deposited on the resorbed surface in isolated areas, thus reconnecting principal fibres of the periodontium. In the third stage, the reparative apposition reforms the normal periodontal attachment, still in a position more distal than the original, resulting in a net distal movement of the socket wall.

Lefkowitz and Waugh (1945) show histologically that the width of the periodontal membrane is maintained during experimental depression of teeth. They state that resorption occurs at isolated areas at different times. At no time are all the periodontal fibres unattached from alveolar bone. They note that areas of resorption are associated with osteoclasts and increased vascularity, and progress slightly further than the average width of the periodontal ligament. Periodontal fibres are then seen to re-establish in new alveolar bone thereby restoring the normal width of the periodontal space.

Enlow (1968) describes depository and resorptive alveolar bone surfaces. Depository surfaces are characterized by successive lamellae of bone incorporating coarse Sharpey fibres. Sharpey fibre bone is formed in those areas of the socket away from which the tooth is moving. Resorptive surfaces are characterized by an eroded, scalloped margin and are located in areas towards which the root is moving.

Kraw and Enlow (1967) describe the process maintaining fibrous attachment on resorptive periodontal bone surfaces.. They observe firstly that osteoclasts advance into the bone, closely followed by vascular channels. Beneath osteoclasts, the fibrous attachment is

severed and is maintained only to the vascular sheath. However, between the osteoclastic Howship lacunae, they report that fibrous continuity is not destroyed. They observe that osteoclasts leave some intrinsic fibres protruding in some areas. Hancox (1972a, 1972b), also states that the actual edge of bone is difficult to define and often appears frayed. Kraw and Enlow (1967) consider that some of these protruding intrinsic fibres may become involved in reattachment of periodontal fibres.

Enlow describes how, when drift has progressed for sufficient time in those areas where the interseptal or interradicular bone is only a thin cortical plate, the entire partition is formed of Sharpey fibre bone. A given point on a periodontal fibre is eventually translocated as a Sharpey fibre through the bone from a depository to a resorptive surface. However, according to Enlow (1968) the Sharpey fibres, previously incorporated at a depository surface, play no part in the attachment of the root associated with the resorptive surface. Attachment to the resorptive surface is by the processes described above.

Baron (1973a) describes a similar process of Sharpey fibre incorporation at depository bone surfaces. Maintenance of sufficient fibre attachment at resorptive surfaces is seen to be achieved by resorption and reattachment by spot deposition proceeding concurrently in different areas.

THE INTERMEDIATE PLEXUS

Sicher (1923) first uses the term 'intermediate plexus' to describe a splicing of alveolar bone fibres to cementum fibres in the

middle of the periodontal ligament. He describes it as a functional adaptation to continuous eruption of the teeth. Eccles (1959), Miura *et al.* (1970) and Orban (1972) confirm the appearance of a plexus during the prefunctional phase of eruption. However, most authors dispute its appearance in the functional periodontium claiming that fibres can be traced the full distance across the periodontal ligament (Bernick, 1960; Trott, 1962; Zwarych and Quigley, 1965; Quigley, 1970). Orban (1976) however, emphasises that despite seeming lack of histologic support for the intermediate plexus, the observed rapid turnover of collagen in the ligament must continually remove some fibrils and replace them with new ones, an alteration that does not necessarily re-establish continuity in the old orientation.

Mouse molars, in common with the molars of man, are classified as teeth which erupt continuously with their investing tissues and without growth of the teeth other than cementum deposition (Ainamo and Talari, 1976). Gianelly and Goldman (1971) state that such movements require a zone of remodelling to continuously maintain the observed functional orientation and spatial configuration of the periodontal ligament. Zwarych and Quigley (1965) reason that with continuous eruption of teeth, if there was no zone of remodelling, a change in orientation of oblique fibres to horizontal fibres would be expected as eruption proceeds. They report no such observation, neither does any subsequent report to date. Furthermore, these workers state that the periodontal fibres must elongate to maintain attachment of a tooth as it moves away from the bone.

Reitan (1967) shows that periodontal fibres under tension become surrounded by bone spicules as the tooth moves, in order to maintain the membrane width. As a bony surface advances in conjunction with physiologic tooth migration the periodontal fibres become buried in the bone (Stein and Weinmann, 1925). This develops a pattern as reported by Zwarych and Quigley (1965), Enlow (1968) and Baron (1973a), in which Sharpey fibres are buried more deeply on a depository surface than on a resorbing surface.

Cohn (1972b) discusses the possible regions where remodelling may occur subsequent to movement of teeth. He states that on speculative grounds, the most likely position of adaptation would be within the periodontal ligament. However, he recognises the very labile nature of alveolar bone and states that remodelling could occur in this region. He concludes that the presence of continuous transalveolar fibres indicates that remodelling occurs within the periodontal ligament. Herman and Richelle (1961) state that if bone collagen is to be replaced without bone resorption, the process must be very slow.

There is much support for the concept of an intermediate plexus despite failure of histologic studies to locate or identify it. Fava-de-Moraes and Villa (1969) report histologic and histochemical changes in the periodontal ligament of rats following starvation for various time intervals. After the loss of 12% of body weight in the rats, they observe slight disorganisation of the collagen fibres. At 20% loss of body weight the principal fibres of the middle part are almost completely disorganised and without definite orientation. It is stated that the cementum Sharpey fibre bundles are much more stable.

Kraw and Enlow (1967) describe the collagen of the middle part of the ligament as immature because it exhibits a different reaction to histologic staining. Hunt and Paynter (1959) show disruption of the central part of the periodontal ligament in Vitamin C deficient guinea pigs. Burkland *et al.* (1976) show that rupture of the periodontal ligament during extraction occurs one third of the distance between the alveolar wall and the cementum, suggesting that this is an area of weakness.

Ten Cate and Deporter (1975), Ten Cate (1976) and Ten Cate *et al.* (1976) describe the fibroblast as not only the cell responsible for formation of collagen, but also as responsible for its breakdown and turnover in the periodontal ligament. Their hypothesis is based on observed turnover of collagen and the great preponderance of fibroblasts within the periodontium. In their electron microscope studies, they observe degraded collagen fragments within fibroblasts. Garant (1976) also finds that fibroblasts contain numerous intracellular or cytosegregated collagen fibrils which appear to have been broken down within the cell.

AUTORADIOGRAPHIC STUDIES RELATED TO THE PERIODONTIUM

Autoradiographic studies uniformly show fast turnover of the radioactive amino acids proline, glycine and lysine in the periodontium, compared to skin and other tissues (Stallard, 1963; Crumley, 1964; Carneiro, 1965; Carneiro and Fava-de-Moraes, 1965; Carneiro and Leblond, 1966; Anderson, 1967; Skougaard, Frandsen and Baker, 1970; Skougaard, Levy and Simpson, 1970; Skougaard and Levy, 1971, Kameyama, 1973, 1975; Page and Ammons, 1974; Rippin, 1976, 1978).

The above studies are based on prelabelling animals with radioactive amino acids and assessing by quantitative autoradiography the rate of loss of radioactivity which is then equated with the rate of turnover of collagen. The observation of rapid turnover of collagen provides an alternate mechanism for remodelling in the periodontal ligament rather than an intermediate plexus spliced with muco-polysaccharide cement (Orban, 1972).

Collagen contains 20-25 per cent of proline or its derivative hydroxyproline (Hausmann and Newman, 1961; Stallard, 1963; Carneiro, 1965; Eastoe, 1976). Carneiro and Leblond (1965) report that 50 per cent of the observed radioactivity in their sections of mouse periodontium is resistant to collagenase suggesting that other moieties involving proline may be present. Their study indicates that grain counts may trace more than just collagen in the periodontium. They emphasise that autoradiographic results must be interpreted cautiously.

Skougaard, Frandsen and Baker (1970) report an investigation into the link between grain counts and biochemical assays of proline and hydroxyproline in squirrel monkeys. They stress that it is difficult to extract the collagen of the periodontal membrane for quantitative analysis. They take an accessible collagen pool (skin) and examine the effects of glucocorticoid administration, which is known to inhibit collagen turnover within this area, by both biochemical and autoradiographic methods. They find that counting of grains on autoradiographs shows quantitatively a similar inhibition as biochemical methods. Furthermore, there is a proportional

inhibition, as measured by grain counts, in the periodontium. They extrapolate these results to accept grain counts in the periodontium as being a reasonably valid parameter for measuring collagen activity.

Rippin (1976, 1978) reports the observed uptake and rate of loss of proline in the rat molar periodontium to be characteristic of uptake into just one protein with a specified half life, or into several proteins with the same half life. At least 91-97% of observed radioactivity in histologic sections of tissues is firmly bound to tissue proteins (Droz and Warshawsky, 1963; Orlowski, 1976). Using a highly specific collagenase, Rippin (1976) concludes that the labelled protein is collagen. Furthermore, he shows that the rate of collagen formation varies at different levels of the interradicular and interseptal bone, being fastest at the apical area and least at the crestal area. Compared to other tissues, Rippin claims that the turnover rate is rapid.

No variation in turnover rate across the width of the periodontal ligament is reported by Rippin (1976, 1977). Stahl and Tonna (1977) also find no difference in proline uptake across the width of the periodontal ligament. Rippin (1978) suggests that rather than an intermediate plexus, all collagen fibres in the periodontium are both rapidly maturing and rapidly turning over.

Orlowski (1976) biochemically assays hydroxyproline and proline concentrations in labelled rat incisor periodontal ligament and measures the radioactivity associated with each amino acid fraction. By using a ratio of proline to hydroxyproline of 1.15:1, and assuming hydroxyproline to be limited to collagen, he calculates the proportion

of radioactivity associated with the collagen and that with the non collagenous protein. His calculations reveal only 25% of activity to be in collagen and he concludes that there is a highly active non collagenous component present 24 hours after injection. Hausmann and Neuman (1961) state, however, that proline is bound prior to hydroxylation and incorporation into collagen. They suggest either the presence of a carrier protein on which proline is hydroxylated prior to being incorporated into tropocollagen or that collagen fragments, with excess proline moieties, are initially formed which later become hydroxylated.

Sodek (1976, 1977) reports conflicting results from those of Orlowski (1976). Using a biochemical micro-assay of radioactive proline from labelled rat molar periodontal ligament, Sodek demonstrates a highly efficient and rapid collagen turnover.

Sodek *et al.* (1977) biochemically determine the amount of radioactivity in collagen by measuring the label in hydroxyproline and comparing this to the total radioactivity. Secondly, they use a specific collagenase and confirm their biochemical findings that 90% of the radioactivity is associated with collagen 3 and 24 hours after labelling. They suggest Orlowski's differing figures may be due to contamination of his sample by free blood proteins or a significant difference between rat molar and incisor periodontal ligament.

Sodek (1976) measures the incorporation of radioactive proline into the salt soluble and insoluble collagen compartments after varying time intervals up to 27 hours by amino acid analysis. He finds a higly efficient (100%) maturation of soluble to insoluble

collagen in periodontal ligament and alveolar bone, in contrast to values of 51% and 32% for gingiva and skin respectively. The rate of collagen formation and, in the non growing animal therefore, the presumed rate of turnover, is also found to be much faster in the periodontal ligament than in gingiva and skin. Sodek (1977) gives values for the half life of mature collagen in rat periodontal ligament of 1 day, in gingiva, 5 days, 6 days in alveolar bone and 15 days in skin corium.

Autoradiography is used also as a topographical bone marker, independent of its role in studying tissue turnover (Tonna, 1974). As a bone marker, tritiated proline has several advantages over other techniques as it is non toxic and free of other non physiologic side effects on cells (Tonna, 1974). Tetracyclines require undecalcified material, allow definition of approximately 10 μm between bands or a space of 2 days between doses and are incorporated into forming crystals. Soft tissue binding does occur and with prolonged administration, calcification is inhibited. Radioactive proline, however, due to its low energy and its incorporation into the collagenous matrix of calcification, is maintained in routine decalcified sections and allows far greater definition of areas of bone formation. It is not lost from the matrix until bone is resorbed and does not accumulate in cell nuclei, thereby limiting any potential for radiation damage (Tonna, 1974).

Tonna (1976b) reports the rates of bone formation in different areas of the alveolar bone of mice ranging in age from 35 days to 104 weeks using tritiated proline as a topographical bone marker.

Daily rates of formation between 2.13 and 4.56 μm are found for 35 day old mice. The technique appears accurate for assessing rates of matrix formation, although interpretation of numbers of silver grains on autoradiographs is questionable (see discussion Ch. 6).

CHAPTER 4

METHODS AND MATERIALS

PREAMBLE TO SELECTION OF THE EXPERIMENTAL METHOD

The anatomical arrangement of transalveolar fibres in the mouse mandible is reported from studies using histologic preparations and light microscopy (Cohn, 1970, 1972a; Quigley, 1970; Dunstan, 1975). The average size of Sharpey fibres is 5 μm (Jones and Boyde, 1974). Sharpey fibres are bundles of smaller fibres which themselves are aggregations of collagen fibrils. These smaller fibres are approximately 0.2 μm in diameter and are at the limit of the resolving power of the light microscope. Collagen fibrils are aggregations of tropocollagen molecules and show the characteristic cross banding of collagen when viewed in the electron microscope (Melcher and Eastoe, 1969). Although electron microscopy is necessary to specifically identify collagen, the technique is not suitable for the relatively low power examination of structural organisation of collagen fibre bundles.

Development of structures can be studied by many techniques. Histologic examination with serial sectioning allows a three dimensional reconstruction of tissues whilst enabling visualization of cellular detail. This method was chosen in the present study as it allows a direct comparison of the results with those of previous studies of transalveolar fibres in the mouse. Furthermore, the technique can be

combined with autoradiography to determine the rate of collagen turnover (Rippin, 1976, 1978).

Cross sectional studies of development involve sacrifice of animals at different ages. To minimize the effects of individual variation and errors of bias, mice of the same sex, strain and bred in the same laboratory were used throughout the project. Experimental and control animals were randomly selected in all groups.

Turnover studies of collagen are based in three categories; histologic, autoradiographic and biochemical. The histologic approach relies on differential staining of young and mature collagen (Herovici, 1965; Miura *et al.*, 1970). The justification for the claim of differential staining is based on morphological observations that young collagen stains blue and mature collagen red with the specific Herovici (1965) technique. The technique is not quantitative and may be considered poorly qualitative as well. This criticism is well grounded as it is reported that differences in tension of skin collagen may also alter its staining reactions (Craik and McNeil, 1966). At the ultrastructural level, evidence of turnover is provided by the demonstration of intracellular breakdown products of collagen (Ten Cate, 1976; Garant, 1976).

Autoradiographic methods are most frequently employed in studies of collagen turnover. The protein collagen is built of three intertwined helices of polypeptides (Melcher and Eastoe, 1969; Eastoe, 1976). The sequence and ratio of the amino acids in collagen is highly characteristic. Glycine, the simplest and smallest of the amino acids comprises one third of the total, and occupies every third

position on the chain (Melcher and Eastoe, 1969). Proline comprises 12% and hydroxyproline 9% of the residues (Eastoe, 1976).

Hydroxyproline is not found in any other protein and this amino acid can therefore be used as a direct biochemical marker of collagen (Sodek, 1976). Hydroxyproline is, however, formed only by hydroxylation of proline, either prior to or after its incorporation into the collagen molecule. It is not taken directly from the blood stream (Stallard, 1963).

All amino acids except hydroxyproline are found in the non-collagenous proteins of the periodontal ligament. Radioactive proline was selected as a label for collagen in this study because the combined ratio of proline and hydroxyproline in collagen, relative to the non-collagenous protein, is the most favourable of all amino acids.

Autoradiographs of histologic sections provide a cumulative record during the exposure period of radioactive emissions. Latent images are formed in the nuclear emulsion which are subsequently developed and may be observed and counted in either the light or electron microscope. Boren *et al.* (1975) show the technique to be extremely sensitive and consistent when correct procedures are followed. The technique has several shortcomings, however. Firstly, re-use of proline by the tissues is significant (Jackson and Heininger, 1974; Sodek, 1976). Secondly, proline is not a specific label for collagen. Other non-collagenous proteins are also labelled and there is no way of differentiating silver grains from these sources and those from collagen in the light microscope (Orlowski, 1976). Autoradiographic techniques, although standardised, have a large number of factors

which may introduce variations to the results, independent of the tissue being examined. Such variables include vascular absorption from intraperitoneal injection, fixation, decalcification, section thickness, emulsion sensitivity, exposure time, temperature and relative humidity, development, fixation and post-staining (Rogers, 1973; Boren *et al.*, 1975). Quantitative autoradiography thus depends on statistical evaluation of results and requires a sufficiently large sample to provide meaningful data.

The biochemical isolation of collagen and determination of specific activity of radioactive hydroxyproline provides a more quantifiable measure of collagen turnover although precise anatomical identification is lost (Sodek, 1976, 1977). For this reason biochemical methods were unsuitable for use in the present study as it would have been impossible to determine whether acitvity was due to surface bone activity or intraosseous fibre remodelling. Moreover, the technique measures decay rates of the samples over short time intervals and may not be as sensitive as the cumulative grain score of autoradiographs.

EXPERIMENTAL METHOD

White, male mice, in several age ranges, were obtained when required from the Waite Agricultural Research Institute breeding stock. The animals were fed mouse cubes and water *ad libitum* and maintained in an animal house with regulated temperature, humidity and lighting.

Experimental animals were injected intraperitoneally with 5 μCi per gram of body weight of L-[3,4(n)-^{3}H] proline; specific activity 40 Ci/mmol, (The Radiochemical Centre, Amersham, England TRK. 439, Batch 7). The stock solution was diluted with sterile isotonic saline to give an injected volume of between 0.3 and 0.5 mls (Appendix I), which was then administered through a 26 gauge needle on a 1 ml tuberculin syringe. Sterile syringes and needles were used for each animal.

The intraperitoneal route was selected as, apart from its relative simplicity, it is known that a moderately high blood level of proline is rapidly achieved (Ross and Benditt, 1965). Carneiro and Fava-de-Moraes (1965) report that following a single intraperitoneal injection of radioactive proline in the mouse, maximum incorporation of labelled material into extracellular products is observed at 4 hours and that thereafter it decreases until virtually none remains at 45 days, except in bone matrix. Control animals were injected with either sterile saline or a similar concentration of L-proline (Biochemistry Department, The University of Adelaide) diluted to an equivalent concentration (Appendix I).

The initial experiment utilized four 23 day old mice, two of which were injected with tritiated proline (5 μCi/gram of body weight), the other two serving as controls (Appendix II). The animals were killed four hours following injection. The 23 day old mice did not show the anticipated anatomical arrangement of transalveolar fibres (Cohn, 1972a; Dunstan, 1975), and the initial experiment to determine if label was incorporated into transalveolar fibres was therefore repeated on older mice.

In the second experiment, four 42 day old mice were used. Two received intraperitoneal injections of tritiated proline, whilst two received L-proline as controls (Appendix III).

Four 42 day old mice were obtained for a third experiment in which the radioactive label was used as a vital topographical bone marker. Two received injections of tritiated proline and two of L-proline into the peritoneal cavity. Following the injection, the animals were kept in separate cages. One experimental and one control animal were killed at each of five and nine days after the injection (Appendix IV).

Development of the supporting structures of the mandibular molars was examined histologically in serial sections of mice aged 10, 14, 16, 18, 20, 23, 28, 42 and 70 days.

TISSUE PROCESSING

The animals were killed by cervical fracture. The heads were immediately removed and placed in ten per cent buffered neutral formalin for a minimum of 24 hours. After this time, the paired mandibles were dissected out, hemisectioned and placed in fresh ten per cent buffered neutral formalin for a minimum of 48 hours. Mandibles were stored at this stage for varying time intervals. Subsequently the left and right sides were processed independently.

Further processing initially involved decalcification. Two techniques of decalcification were used as it is reported that this step may affect the quantity of radioactive material retained in the tissue (Beertsen and Tonino, 1975). These workers favour

decalcification in EDTA. Non-radioactive material and selected radioactive material (Appendix II and IV) was decalcified in formic formate decalcifying solution (Culling, 1974). The remaining radioactive material and controls were decalcified in 10% EDTA with 7.5% polyvinylpyrrolidone buffered to pH 6.95 at 4°C. Radiographs were taken to determine the stage of decalcification. Following decalcification in formic formate, the mandibles were washed in neutralizing solution for 24 hours. Those decalcified in EDTA were placed for 24 hours in fresh buffer, pH 6.95.

The decalcified mandibles were trimmed prior to further processing to facilitate orientation of the blocks for section cutting. A sharp blade was placed against the lingual cortical plate adjacent to the molar teeth and parallel to the lingual cusps of the teeth. The incisor was cut through in this plane leaving a flat surface which was later placed against the floor of the mould during embedding. The material was subsequently passed through graded alcohols and double embedded in celloidin and paraffin according to the technique of Peterfi (Culling, 1974) prior to being blocked in paraffin (Paraplast) in the Tissue Tek II Tissue Embedding Centre (Lab-Tek Products, Naperville, Illinois).

SECTION CUTTING FOR LIGHT MICROSCOPY

The tissue blocks were cut at 5 μm on a rotary microtome (Leitz Wetzlar, Midland, Ontario) and serial sections, from the lingual to the buccal side, placed on chromic acid cleaned gelatinised slides. The plane of section was determined by the orientation of the tissue

block in the mould and the orientation of the block on the microtome relative to the knife. Every effort was made to obtain true mesiodistal sections through the long axis of the teeth. This was not possible, however, as the three molar teeth progressively became more lingually inclined from the mesial to the distal of the jaw. There was a similar problem orientating the blocks for sections in the horizontal and buccolingual planes. To help orientate the reader, schematic diagrams indicating the plane of section and the depth from which a given illustration has been taken, have been included with the necessary photomicrographs.

AUTORADIOGRAPHY

Autoradiographs were made of selected slides of the radioactive material as well as some control slides using the dipping technique of Rogers (1973, p.310-312). Negative chemographic control slides, with stained and unstained sections were also included. The slides were deparaffinized and hydrated prior to being dipped in nuclear research emulsion (Ilford K2). The emulsion was placed in a coplin jar in a 43°C water bath. It was then diluted 1 to 1 with distilled water and 1% glycerin added as a plasticizer. The dipping procedure was carried out under a safe light and at 50% relative humidity. After dipping, the slides were drained, their backs wiped and then placed on a cold metal surface to gel the emulsion. When gelled they were transferred to a bench to dry in the dark for 2 hours. They were then placed in light tight slide boxes with silica gel (Drierite) in the refrigerator at 4°C to expose. Boren *et al.* (1975) claim latent image fading to be negligible at 4°C. Exposure was routinely

for five weeks. The slides were then taken to a photographic darkroom, transferred to glass racks, developed in Kodak D19 or Ilford Phen-X developer and fixed in 30% sodium thiosulphate (Rogers, 1973).

HISTOLOGIC STAINS

Stains were used both before dipping and after developing autoradiographs, and on the other material in the study in order to differentiate various structures for photographic and visual interpretation. Stains for this study were primarily required to show collagen. A further requirement was that they should differentiate Sharpey fibres from bone matrix. Both of these structures have a large collagenous component, although they vary in the arrangement of the individual collagen fibres (Jones and Boyde, 1974). It was also desirable that the staining results be reproducible on autoradiographs. Recognized collagen stains, as well as several other stains were evaluated in this study for use on normal sections and on autoradiographs.

Silver impregnation with the method of Gordon and Sweet (Culling, 1974) was used for some slides. However, the technique was considered unsuitable for staining autoradiographs, which themselves rely on silver grains to mark sites of radioactive decay.

Van Geison stain (Luna, 1968) was used for some slides and was also evaluated for post-staining autoradiographs. Weigert's haematoxylin and eosin (Luna, 1968) was also used both for the autoradiographs and on other slides to identify nuclei. Stains used

prior to dipping autoradiographs were aldehyde fuchsin (Fullmer and Lillie, 1958) and Pollack's trichrome (Luna, 1968, p.116-117).

Many stains were used over processed autoradiographs. Various combinations of 1 per cent aqueous solutions of sirius blue, sirius red, safranin O, beibrich scarlet and coomassie blue were evaluated in an attempt to clearly differentiate Sharpey fibres in autoradiographs. Selected pairs of solutions were sequentially flooded over deparaffinized slides. Sections were then dehydrated through alcohol and mounted in di-butyl phthallate in xylol (pix).

Other techniques using toluidine blue (Luna, 1968), light green (Luna, 1968), metanil yellow (Luna, 1968) and Pollack's trichrome (Luna, 1968) were also used, both over normal sections and autoradiographs. Some toluidine blue stained sections were mounted in water.

Thurston and Joftes (1963) list the stains most suitable in their hands for staining autoradiographs of mouse tissue. Of these, the nuclear fast red, indigocarmine and picric acid technique of Mortreuil-Langlois (1962) was evaluated as indigocarmine has previously been used to show Sharpey fibres in histologic sections (Weidenreich, 1923; Smith, 1960).

SPECIAL MICROSCOPIC TECHNIQUES

Phase Contrast

Optical methods of obtaining contrast in sections were thoroughly evaluated. Phase contrast effects are achieved by diffraction of

light by tissue components (Culling, 1974). Both stained and unstained tissue could therefore be examined with the phase contrast microscope.

Polarized Light

Collagen is anisotropic with a single optical axis parallel to the long axis of the collagen molecules (Schmidt and Keil, 1971). Structural differences in collagen orientation are therefore visible in the polarising microscope. This method was investigated briefly but required special facilities which were not practicably available.

Differential Colour Illumination

Differential colour illumination using the Rheinberg method (Gustav, 1976) was also tested. A magenta filter was so placed in the light path of the transmitted light microscope, that it completely obstructed the primary light path passing through the objective aperture, in a similar manner to the opaque disc of a darkfield condenser. A green filter was placed around the red disc so that the specimens were viewed under direct red light and peripherally refracted green light.

Differential Interference Contrast

This particular optical system is a relatively recent advance. Culling (1974) describes its appearance in the biological laboratory after 1945. Even so, he describes it as a complex tool of limited advantage over a conventional phase contrast microscope. A modern design of this system which was simple to use was available for a

short time during the study (Courtesy ANAX Pty. Ltd., Adelaide, South Australia). Instead of relying on the interference between two out-of-phase images of the same point to build up a light and dark image, as in the phase contrast microscope, this system split the light into two separate parallel out-of-phase paths. This was achieved by passing the light through a Wollaston Prism. The two separate light rays which originated from the same source were recombined in a second Wollaston prism above the object. If the two parts of tissue that the split rays passed through were of different optical refractive power, a phase difference was produced resulting in interference. Consequently each point on the final image was compounded from two parallel light rays which had followed different paths in the tissue. An image of optical thickness was thus created with a three dimensional effect.

THE SCANNING ELECTRON MICROSCOPE

Greater resolution was obtained on selected sections by examination in the scanning electron microscope. Coverslips were removed and the sections washed in xylol followed by 2 changes of absolute alcohol, each for 1 hour, then placed in absolute alcohol over anhydrous copper sulphate for 1 hour before being air dried. The slides were then cut so that each section was held on a piece of glass less than 1 cm x 1 cm. These glass pieces were mounted on aluminium stubs and coated with gold in an inert atmosphere with a sputter coating technique using a Mini Coater (model MCE 200, Commonwealth Scientific Company, Alexandria, Virginia). The material was examined in the scanning electron

microscope (Seimens Autoscan ETEC Inc., U.S.A.) operated at 10 or 20 kilovolts and photographs recorded on Ilford FP4 120 roll films.

PHOTOGRAPHIC PROCESSING

Stained and unstained slides were examined with the transmitted light microscope. Photomicrographs were taken on the Zeiss Axiomat on Ilford FP4, 5" x 4" plate film or 35 mm Kodak photomicrography colour film. Some photomicrographs were also taken on an Olympus BH microscope with the differential interference contrast attachments. These photomicrographs were recorded on 35 mm Ilford Pan F film. Exposure was automatically controlled on both machines. A scribed micrometer was photographed to provide accurate statistics of enlargements.

All films were developed according to manufacturers' instructions. Negatives were printed onto Kodak veribrom or Ilford ilfospeed photographic paper to form similar sized single or composite master prints. The master prints were labelled and photographed with Ilford Pan F 35 mm film. From these negatives the final prints were made. This indirect technique was found to provide for consistently clearer and more precise labelling of the photomicrographs. Colour plates were prepared from 35 mm positive slides using the Ilford Cibachrome technique.

CHAPTER 5

RESULTS OF THE INVESTIGATION

HISTOLOGIC STAINING PROCEDURES

Gordon and Sweet Silver Impregnation

The Gordon and Sweet method for silver impregnation (Culling, 1974) did not stain cellular detail but did provide adequate clarity and definition of the Sharpey fibres for observation and photomicrography. Extrinsic, or Sharpey fibres within bone were impregnated with silver and they became distinguished from similarly impregnated intrinsic bone matrix fibres by their orientation into bundles. Counterstaining with a 0.25 per cent aqueous solution of neutral red or light green, improved the contrast for photomicrography (Fig. 3). Silver staining was not compatible with autoradiographs.

Pollack's Trichrome

Pollack's trichrome stain was used in the technique of Luna (1968). The step involving nuclear staining with Giemsa's solution was routinely omitted to enhance the collagen staining. Sections stained with Pollack's trichrome for less than 10 seconds had green-stained collagenous elements in the periodontal ligament, red-stained cells and red-stained bone. Sections stained for more than three minutes had similarly green-stained periodontal ligament with red-stained cellular elements, but most of the bony matrix appeared green. Some areas of

dentine and periosteal bone were still stained red. At an intermediate time interval, however, there was differential staining of the extrinsic and intrinsic bone fibres. Sharpey fibres held the red stain, whilst the surrounding bone was green. Sharpey fibres so stained, became green on entering the periodontal ligament (Fig. 4). The resulting sections gave good contrast for black and white photomicrography. Unfortunately, these results were not reproducible on slides coated with Ilford nuclear emulsion although some definition of fibre patterns was still seen (Fig. 5). Sections stained with Pollack's trichrome prior to dipping in nuclear emulsion lost the stain during the subsequent processing.

Gomori's Trichrome

Gomori's trichrome stain (Luna, 1968) gave similar colours to the tissue elements as Pollack's trichrome but without the differential staining of intrinsic and extrinsic collagen fibres. All bone areas appeared green.

Haematoxylin and Eosin

Haematoxylin and eosin (Luna, 1968) failed to differentiate the Sharpey fibres in bone from the bone matrix, although it stained both autoradiographs and normal sections similarly (Fig. 6). In some autoradiographs a precipitate of eosin was observed in the nuclear emulsion. Only a very light eosin stain was possible without affecting the autoradiographs.

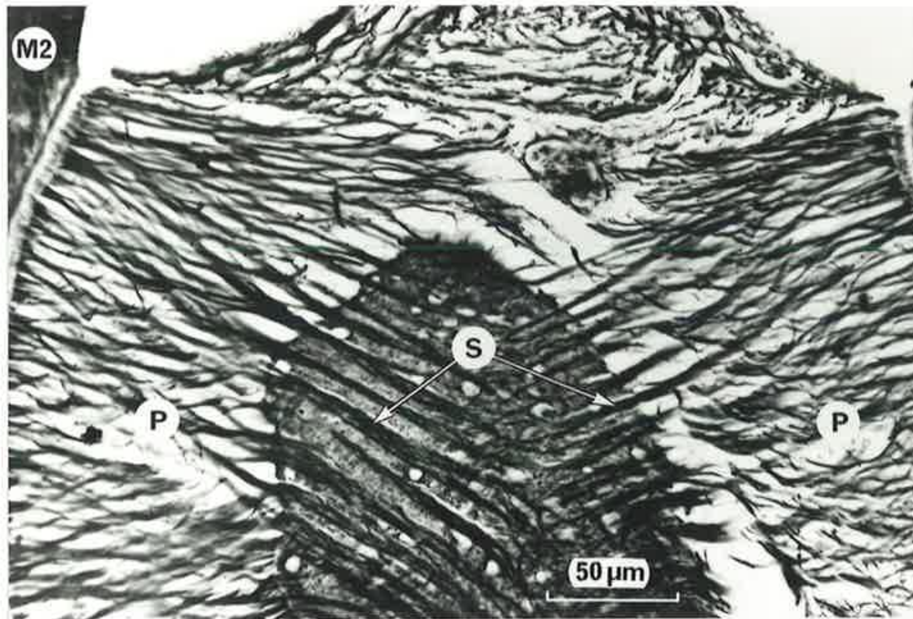

Fig. 3

Mesiodistal section of the mandible of a 23 day old mouse. Although Sharpey fibre bone forms the entire coronal part of the interdental septum distal to the first molar, individual Sharpey fibres do not cross the bone. Fibres from each tooth form a V-shaped pattern in the bone. M2, second molar (mesial root); S, Sharpey fibres in bone; P, periodontal fibres. Gordon and Sweet silver impregnation and neutral red. x250

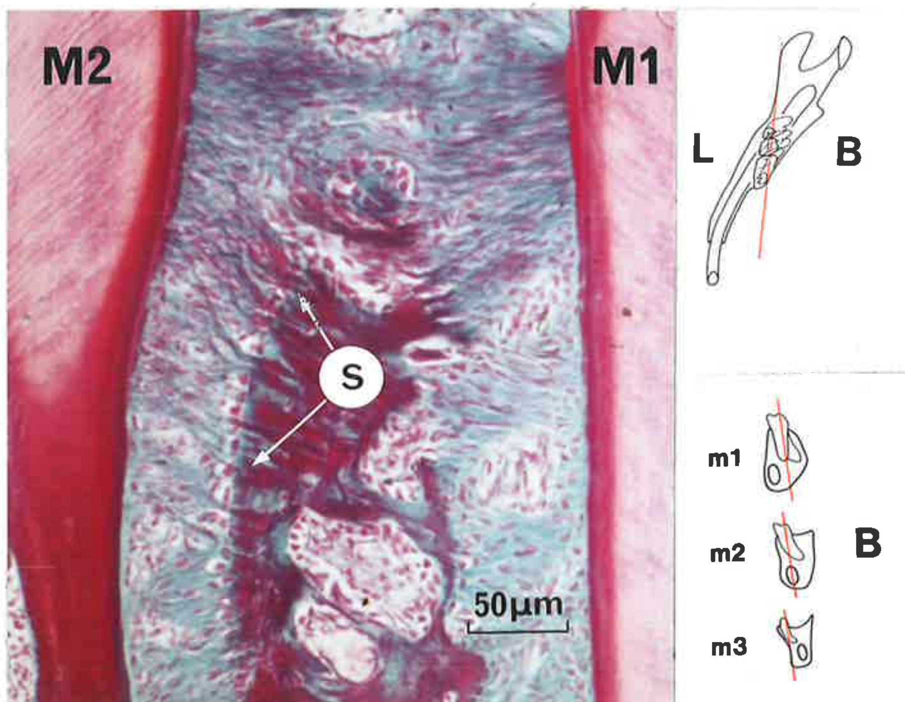

Fig. 4

Interseptal bone distal to the first molar in a 28 day old mouse. Periodontal fibres attached to the second molar, enter the bone as Sharpey fibres and pass to the resorptive surface adjacent to the first molar. M1, first molar; M2, second molar; S, Sharpey fibres. Pollack's trichrome. x100

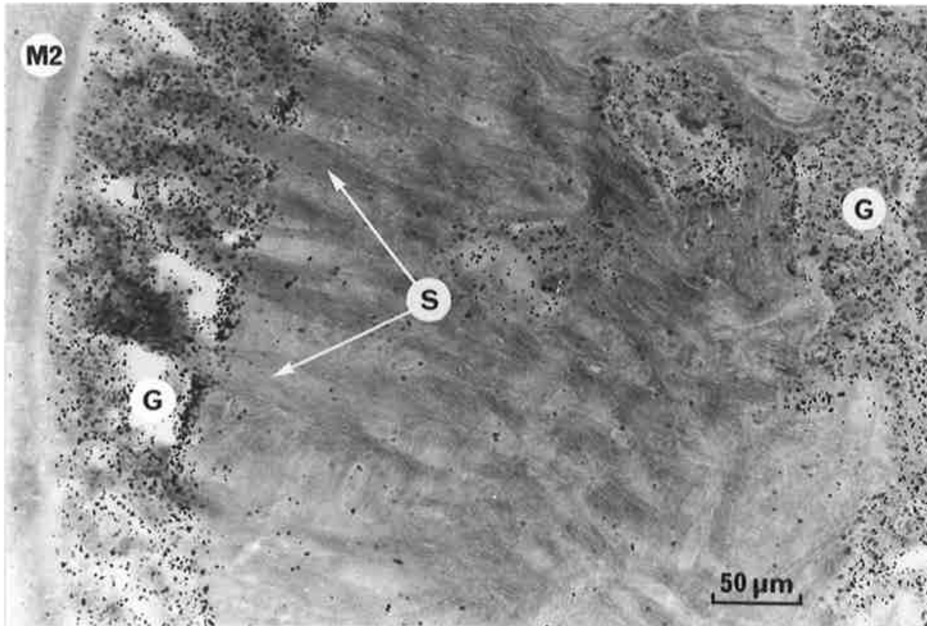

Fig. 5

Autoradiograph of a 42 day old mouse mandible showing the crestal area of interseptal bone distal to the first molar. The dense patches of silver grains over the distal alveolar wall show bone deposition around collagen fibres, incorporating periodontal fibres as Sharpey fibres. M2, second molar; G, silver grains; S, Sharpey fibres in bone. Pollack's trichrome. x250

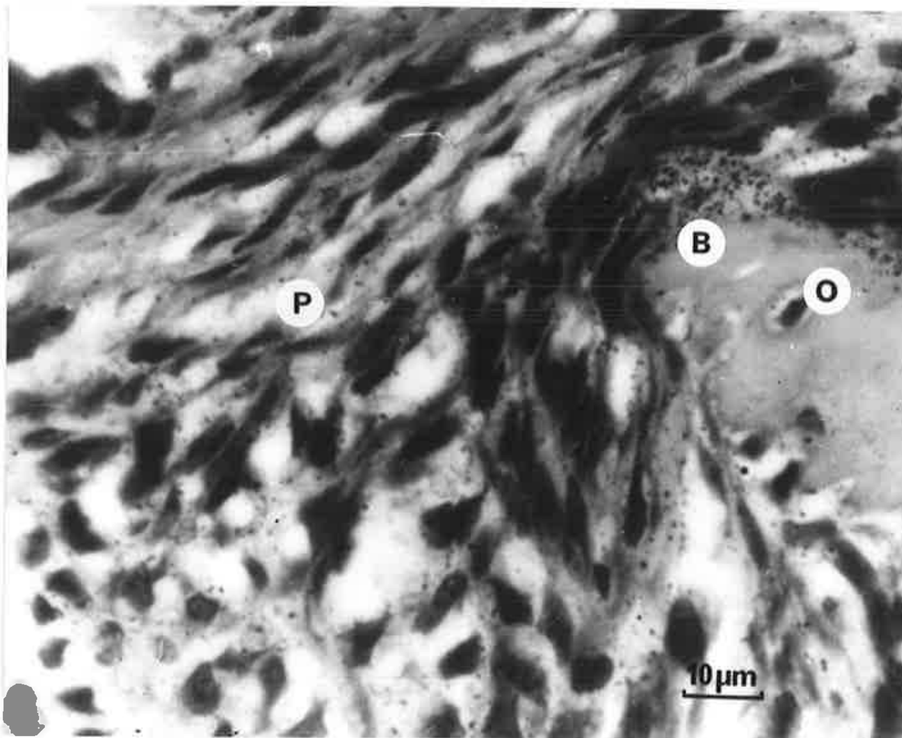

Fig. 6

Autoradiograph of a 23 day old mouse killed 4 hours after labelling, showing silver grains over a forming bone crest and collagen fibres in the periodontal ligament. P, periodontal ligament; B, bone; O, osteocyte. Haematoxylin and eosin. x800

Van Geison's Stain

Van Geison's stain for collagen (Luna, 1968) was also used, but the staining results were inferior to the silver impregnation method and to those seen with Pollack's trichrome. Furthermore, when used over autoradiographs, this stain affected the silver emulsion and reduced the number of silver grains. The stain was therefore considered not useful for post-staining autoradiographs.

Nuclear Fast Red, Indigocarmine, Picric Acid

The nuclear fast red, indigocarmine, picric acid staining technique of Mortreuil-Langlois (1962), was suitable for post-staining autoradiographs. Using a saturated aqueous solution of indigocarmine, Smith (1960) describes the Sharpey fibres in bone from various animals as staining a deeper violet than the surrounding matrix when seen in longitudinal section. In transverse section, he states that Sharpey fibres appear as round pink areas with violet dots. Although the results seen were not as striking as described by Smith (1960), some definition of Sharpey fibres was observed. The technique was very useful as it did not affect the nuclear emulsion (Fig. 7).

Other Stains

Several combinations of neutral stains were used in an attempt to find a method suitable for post-staining autoradiographs, as it was suspected that acid stains, or acid solutions, affected the nuclear emulsion. Sirius blue, over-stained with safranin O, gave some differential staining with red Sharpey fibres and blue matrix. Light

green and sirius red were also used. However, no clear and reproducible results were obtained. Coomassie blue was not useful in showing the areas of interest as it stained the gelatin base of the nuclear emulsion.

Metanil yellow gave reasonable definition of collagen fibres in the periodontal ligament with good contrast for photography of silver grains. Bone matrix, Sharpey fibres and cells, were poorly stained as a yellow background. Acid fuchsin, the dye responsible for the red Sharpey fibres in sections stained with Pollack's trichrome and Masson's trichrome, was used and counterstained with metanil yellow. This resulted in a degree of differentiation of Sharpey fibres (Fig. 8). It did, however, also stain the nuclear emulsion, causing a crazed appearance on some sections.

Light green was found to provide a general light background stain which could be used to improve contrast in conjunction with beibrich scarlet, which stained bone and Sharpey fibres red. The light green displaced the red dye from the bone matrix providing a contrast to the Sharpey fibres in autoradiographs. However, the nuclear emulsion was also deeply stained with beibrich scarlet resulting in a loss of definition.

Prestaining autoradiographs with aldehyde fuchsin (Fullmer and Lillie, 1958) was quite successful. Silver grains were not associated with oxytalan fibres and, apart from strong staining of reversal lines in bone, the technique did not highlight areas or structures of interest.

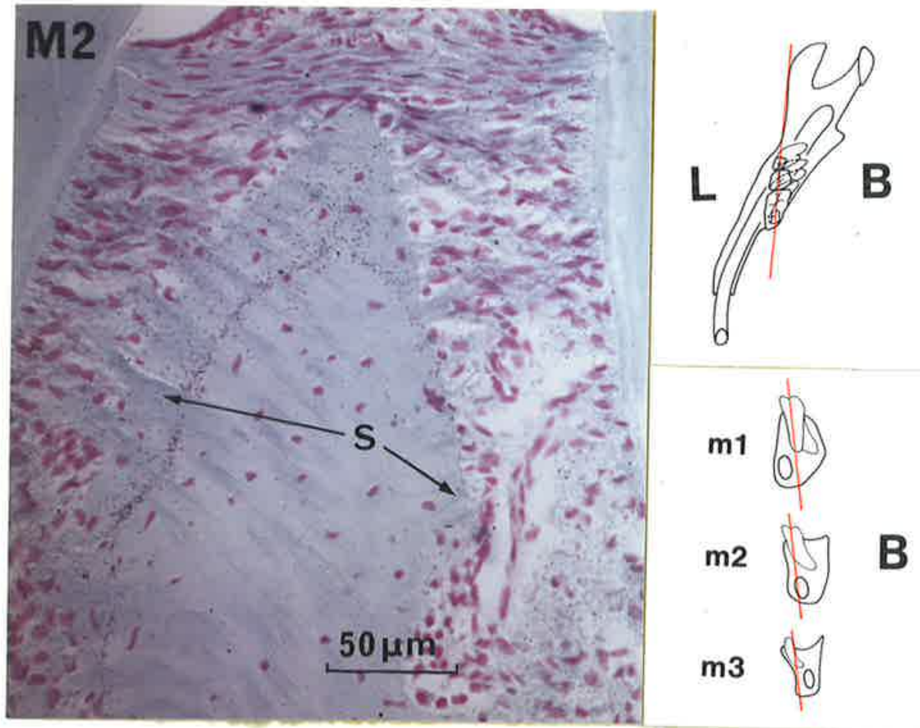

Fig. 7

Autoradiograph of the interseptal bone between the first and second mandibular molars of a 42 day old mouse killed 9 days after labelling. Sharpey fibres associated with the second molar form a greater part of the septal bone matrix than those from the first molar. M2, second molar; S, Sharpey fibres in bone. Nuclear fast red, indigocarmine and picric acid. x250

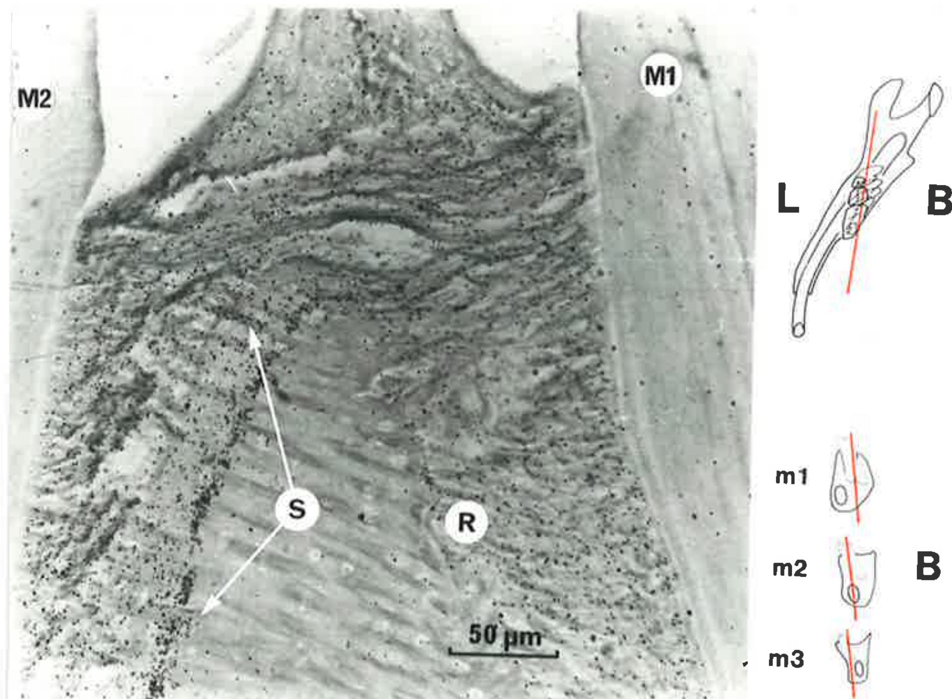

Fig. 8

A serial section from the same molar segment as Fig. 7, taken further towards the buccal. The alveolar surface adjacent to the first molar is resorptive with periodontal fibres attached by spot deposition of bone. New Sharpey fibres have formed at the alveolar crest since the time of labelling. M1, first molar; M2, second molar; S, Sharpey fibres in bone; R, resorptive surface. Acid fuchsin and metanil yellow. x200

Special Microscopic Techniques

Phase contrast images appeared crisp and well defined in both stained and unstained sections. Sharpey fibres could be seen in unstained sections due to their different orientation compared to the intrinsic fibres of the bone matrix. Silver grains were also well defined and appeared as either black or white dots due to the phase halo effect (Fig. 9).

Differential interference contrast was a particularly useful technique for observing structural detail in unstained sections. The images produced had a three dimensional character due to variations in optical thickness of the tissue. This was ideal for distinguishing the Sharpey fibres in bone without the need for histologic staining. Autoradiographs could thus be examined without fear of damage to the delicate nuclear emulsion (Fig. 10).

Differential colour illumination on the thin demineralized sections, with only the imprecise adaptation of filters on the condenser, was found to be very promising for observing autoradiographs. The lateral illumination was reflected by the silver grains, and the background was evenly illuminated with a contrasting colour. No differential optical staining was produced in the tissue (Fig. 11).

Under polarized light unstained collagen fibres showed marked birefringence unless cut perpendicularly to their optical axis (long axis). With polarizer and analyser set at extinction, when the stage was rotated through 90 degrees any given point passed through a cycle from dark to light and back to dark again. Sharpey fibres and periodontal fibres have an irregular course and showed a wave of

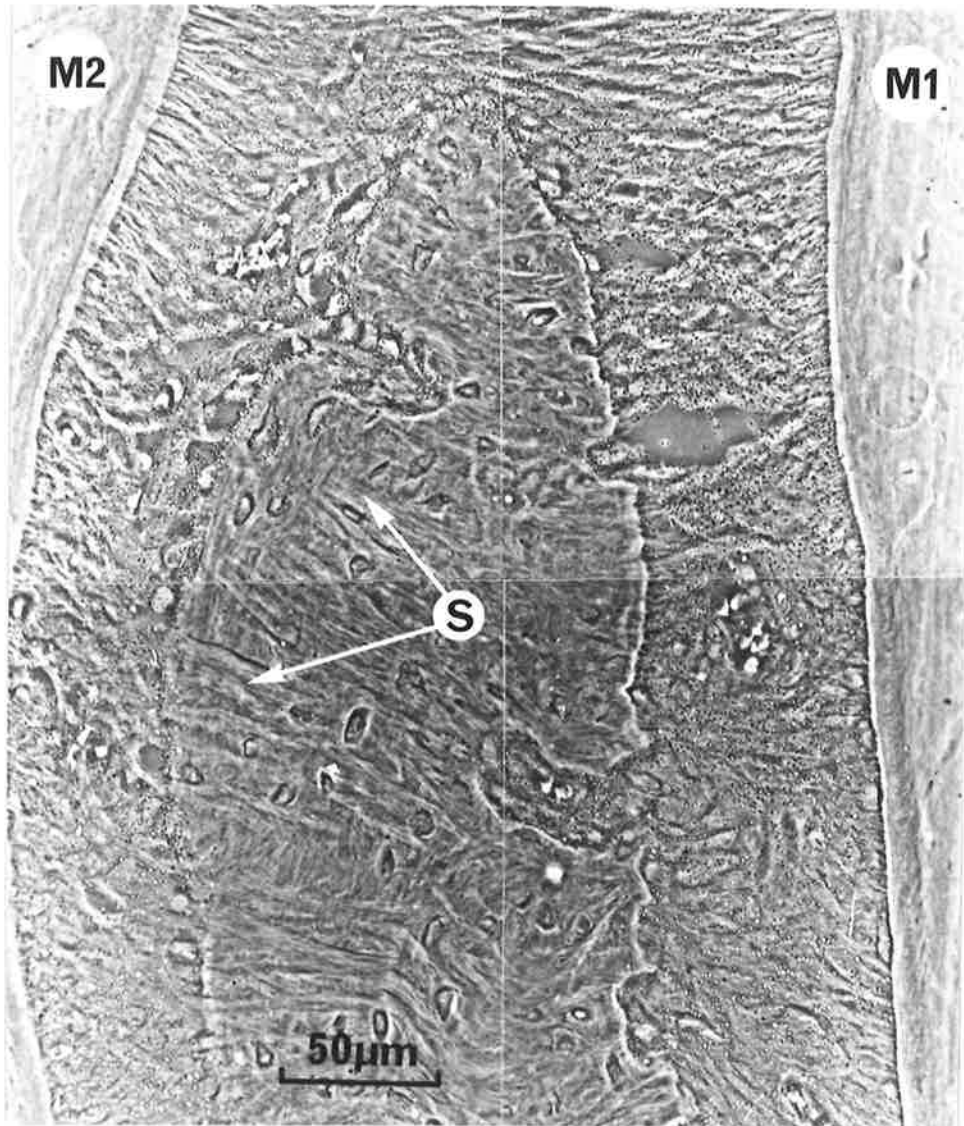

Fig. 9

Composite photomicrograph from a 42 day old mouse killed 4 hours after labelling seen under phase contrast. Silver grains appear black or white due to a phase halo. M1, first molar; M2, second molar; S, Sharpey fibres. Unstained. x500

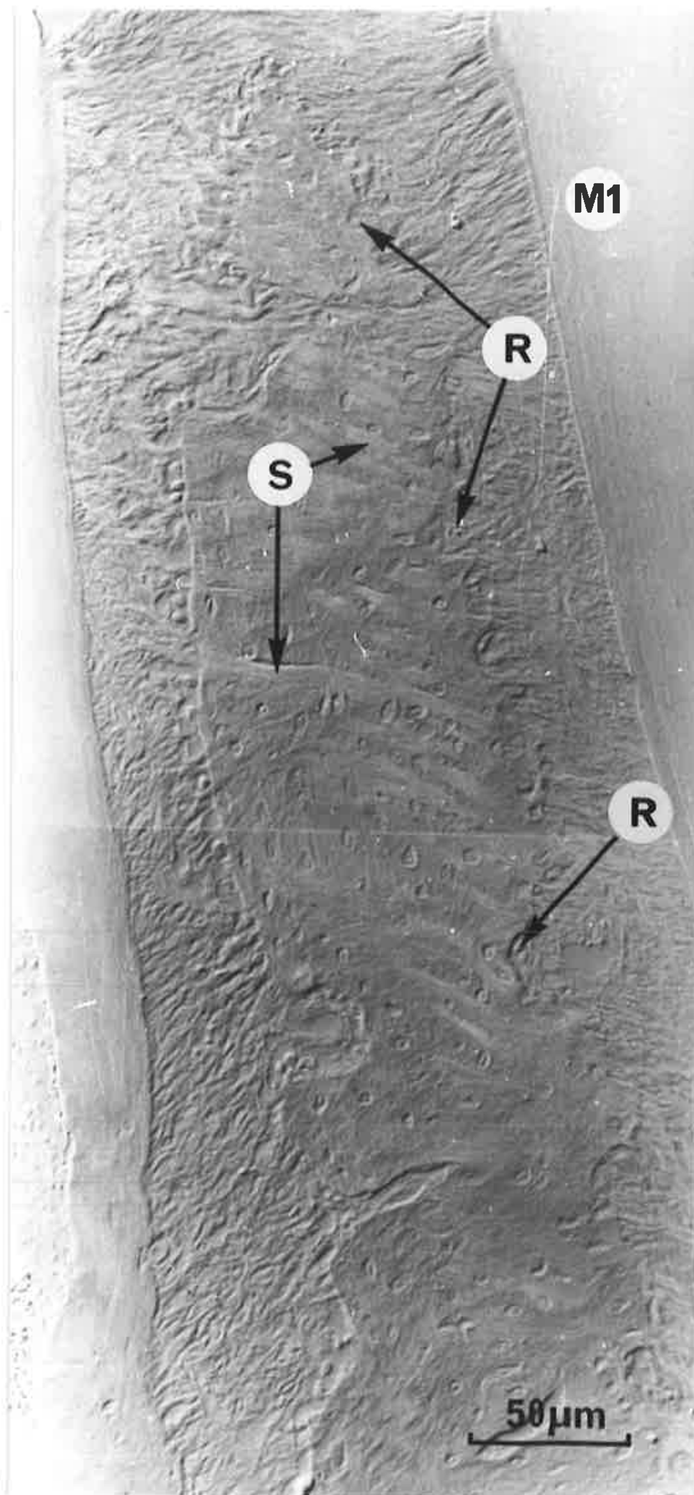

Fig. 10

Composite photomicrograph of an unstained section of a 42 day old mouse seen with differential interference contrast. The three dimensional effect is caused by variations in optical density. M1, first molar; S, Sharpey fibres in bone; R, resorption lacunae. x100

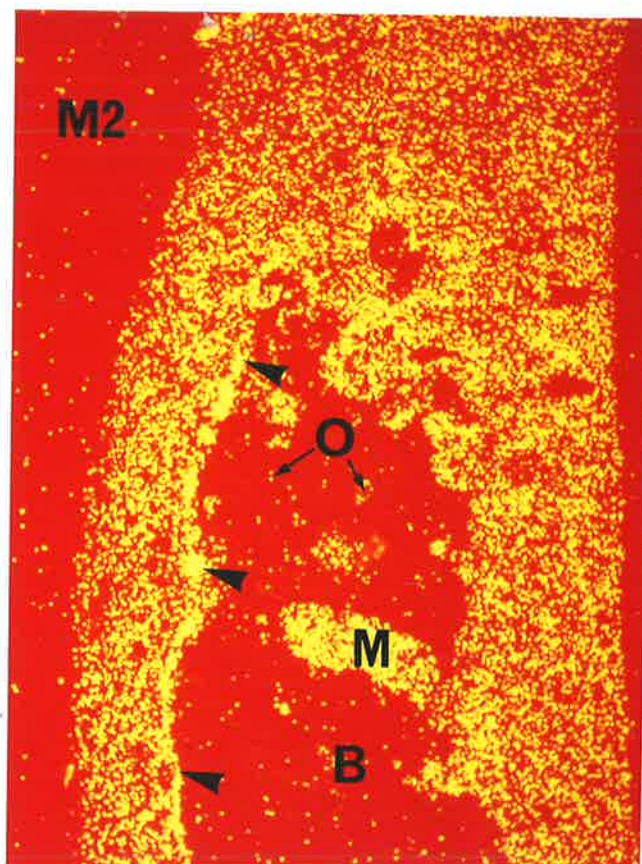

Fig. 11

Unstained autoradiograph seen under Rheinberg's differential colour illumination. The yellow dots are silver grains over a section taken from a 42 day old mouse killed 4 hours after labelling. Note the dense line of silver grains, large arrows, over the distal alveolar surface adjacent to the second mandibular molar.
M2, second molar; O, osteocyte; M, marrow space; B, bone.
x200

undulating extinction when the stage was rotated, Bone matrix surrounding Sharpey fibres in the alveolar crest of adult animals maintained a fairly constant degree of illumination as its intrinsic fibres were randomly dispersed in a plane parallel to the socket surface. Haversian systems have a more organised system of intrinsic fibres, as does lamellar bone, and these areas were strongly birefringent. In the young animals the trabeculae of bone in the developing interradicular and interseptal areas were birefringent but extinguished non-uniformly. Polarized light was unsuitable for demonstrating the spatial relationships of Sharpey fibres for illustration, as in any one position, only those fibres or parts of fibres in one direction could be seen.

AUTORADIOGRAPHIC RESULTS

Three separate autoradiographic experiments were carried out. The first and second were performed on 23 and 42 day old mice respectively and the animals were killed 4 hours after labelling with radioactive proline (Appendices II and III). In the developed autoradiographs from these animals, silver grains were seen over the periodontal ligament, forming bone, osteocytes, and in the pulp and marrow spaces. No silver grain concentrations above normal background levels were observed over deeper bone matrix (Fig. 11). The third experiment utilized 42 day old mice with a delay of either 5 or 9 days between the injection and the time the animals were killed (Appendix IV). In these animals there were fewer silver grains over the periodontal ligament, pulp and marrow spaces. There was also a dense line of

silver grains over some specific sites in the bone, marking the position of the forming bone matrix at the time of injection (Fig. 12). The distribution of silver grains seen in autoradiographs of each of the groups will be described separately.

A. *23 DAY OLD MICE KILLED AT 4 HOURS* *(APPENDIX II)*

Periodontal Ligament

Qualitatively, silver grains appeared evenly distributed across the periodontal ligament attached to each of the roots of the molar teeth (Fig. 13). The concentration of silver grains over the transeptal fibres appeared to be less than over the other areas of ligament. No counts were made of silver grain concentration in the periodontal ligament. Turnover in this region has been reported, (Carneiro and Fava-de-Moraes, 1965; Rippin, 1976, 1978) and was not the subject of this study. The distribution of silver grains observed in the material was, however, similar to that reported by these workers.

Cementum and Dentine

The cementum-periodontium border was marked by the absence of silver grains over both cementum and the deeper dentine. The pulpal margin of the dentine was heavily labelled signifying continuing matrix deposition by the odontoblasts (Fig. 14). Apically, the line of silver grains extended out from the root canal over the forming horizontal face of cellular cementum (Fig. 14). Periodontal fibres were attached only to the lateral, more vertical surface of cellular

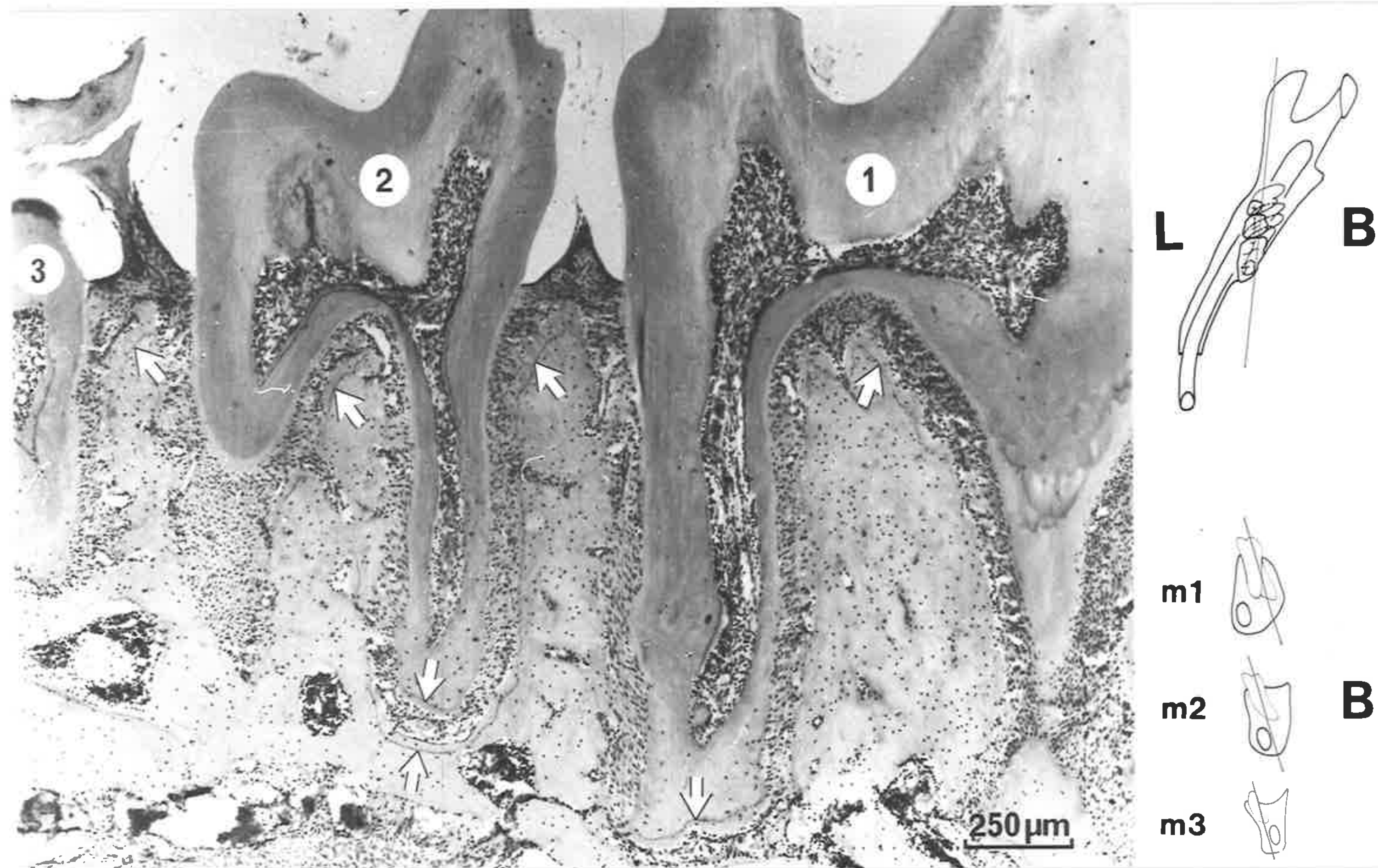

Fig. 12

Left mandibular molar segment of a 42 day old mouse killed 9 days after labelling. The line of silver grains, arrowed, marks topographically the depository surfaces at the time of injection. 1, first molar; 2, second molar; 3, third molar. Nuclear fast red, indigocarmine and picric acid. x40

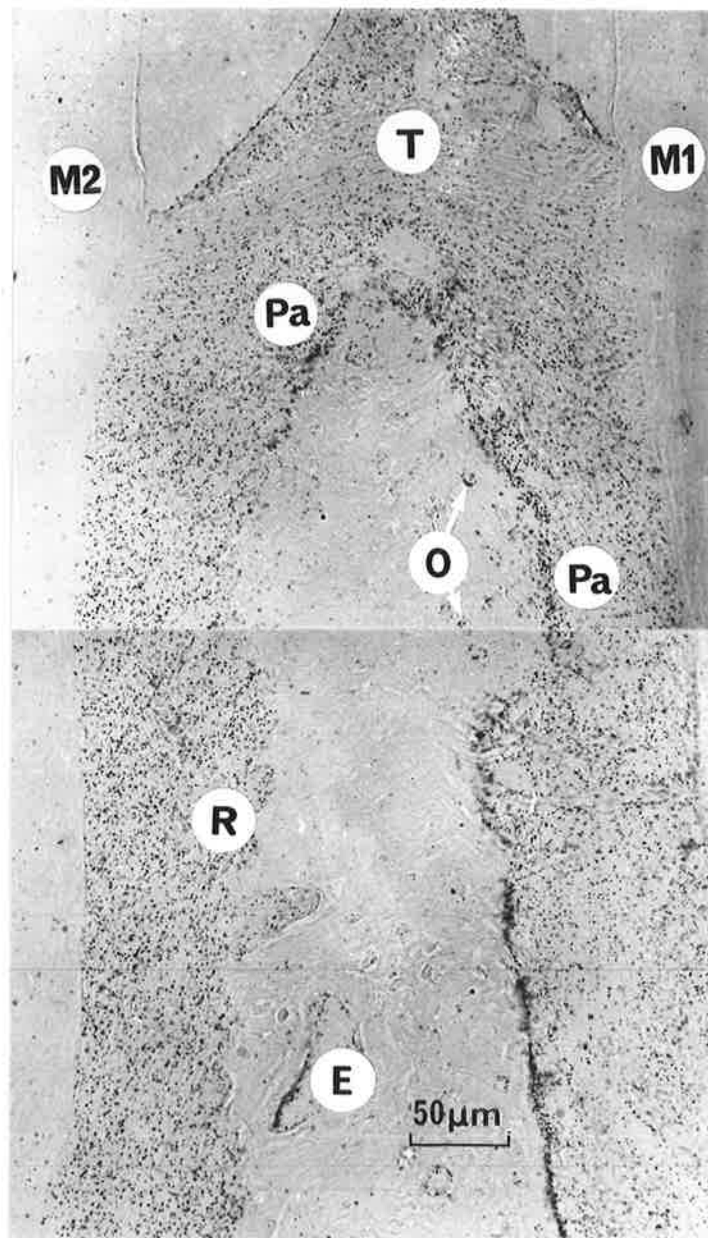

Fig. 13

Unstained autoradiograph of a mesiodistal section from a 23 day old mouse mandible showing the distribution of silver grains 4 hours after labelling. Silver grains above background levels are not seen over the interseptal bone which Cohn (1972a) and Dunstan (1975) describe as incorporating transalveolar fibres. At this age the teeth are moving vertically to occlusion. The apparently anomolous appearance of mesial migration is due to tipping during the rapid eruptive phase. M1, first molar; M2, second molar; T, transeptal fibres; O, osteocytes; R, resorptive bone surface; Pa, periodontal apposition; E, endosteal apposition. x250

cementum, which did not have such a marked concentration of silver grains.

Alveolar Bone

Active areas of bone deposition at the time of labelling were clearly shown on the autoradiographs, appearing as areas of high silver grain concentration. Such areas were only observed on either endosteal, periosteal, or periodontal surfaces of bone. Not all bone surfaces were depository however. Some had the scalloped margin characteristic of active resorption and others appeared to be in a resting phase.

On endosteal surfaces, patches of increased grain concentration were usually seen only on one side of the marrow, or vascular space concerned. It was noted that these areas were often on the side of a space which was adjacent to a resorbing periodontal surface (Fig. 13). Primary Haversian systems were being formed by endosteal apposition of bone in the marrow spaces.

On periodontal surfaces, there was marked activity around the developing third molar and over the alveolar bone crests. Up to 23 days the movement of the teeth was predominantly in a vertical direction. Deposition of bone was observed on both the mesial and distal sides of the interseptal bone between the first and second molars (Fig. 13). A resorptive bone surface in the apical area adjacent to the mesial of the mesial root of the second molar, seen on Fig. 13, was associated with distal tipping of the crown during the rapid eruptive phase.

Over deeper areas of bone matrix, a few silver grains were seen over the edges of some osteocyte lacunae, suggesting that these cells were continuing to function as bone matrix forming cells (Figs. 6, 13). Concentrations of silver grains above background levels were not seen over any other areas of bone matrix, including those areas described by Cohn (1972a) and Dunstan (1975) as containing transalveolar fibres (Fig. 13).

B. *42 DAY OLD MICE KILLED AT 4 HOURS* *(APPENDIX III)*

Periodontal Ligament

An even distribution of silver grains was observed over the entire width of the periodontal ligament around each tooth (Fig. 16). Characteristically, major fibre bundles inserting into the bone were not marked with silver grains at the bone-periodontium interface (Fig. 5). A lower concentration of silver grains over the transeptal fibres was again observed between the first and second molar teeth (Fig. 16) and the second and third molar teeth, than over the rest of the periodontium.

Cementum and Dentine

As described for 23 day old mice, no silver grains above background concentration levels were seen over cementum or dentine, except over the forming margins of predentine and apical cellular cementum (Fig. 15). Patches of silver grains were seen only over discreet areas of predentine rather than over the whole surface. There appeared to be minimal apposition of cellular or acellular cementum on the lateral

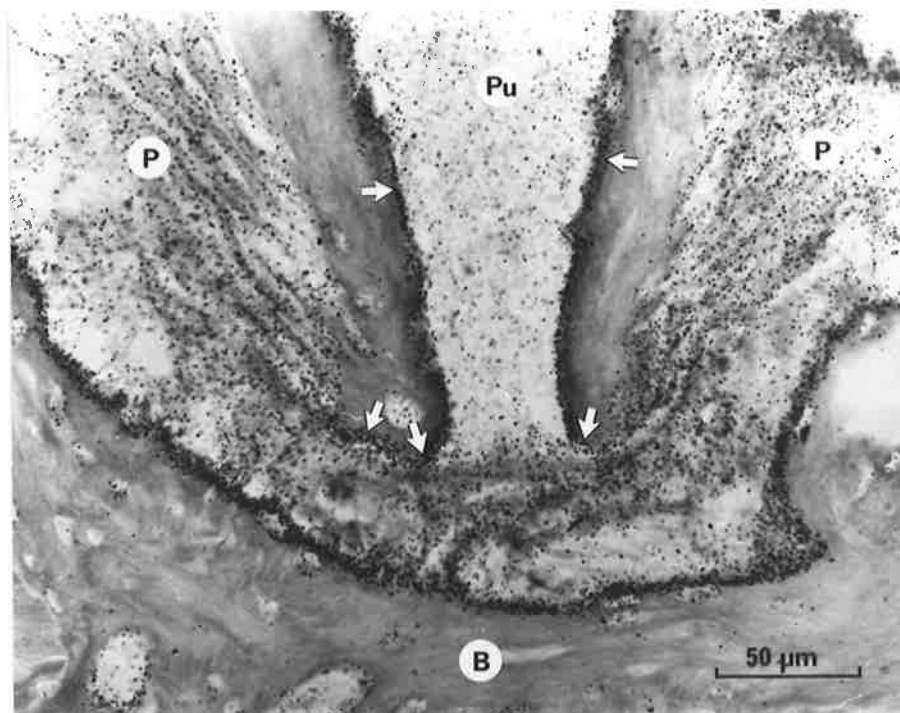

Fig. 14

Autoradiograph of a 23 day old mouse killed 4 hours after labelling. The heavy deposition of silver grains on the pulpal margin of dentine extends out over the apical face of cellular cementum, arrowed. P, periodontal ligament; Pu, pulp; B, apical bone. Acid fuchsin and metanil yellow. x250

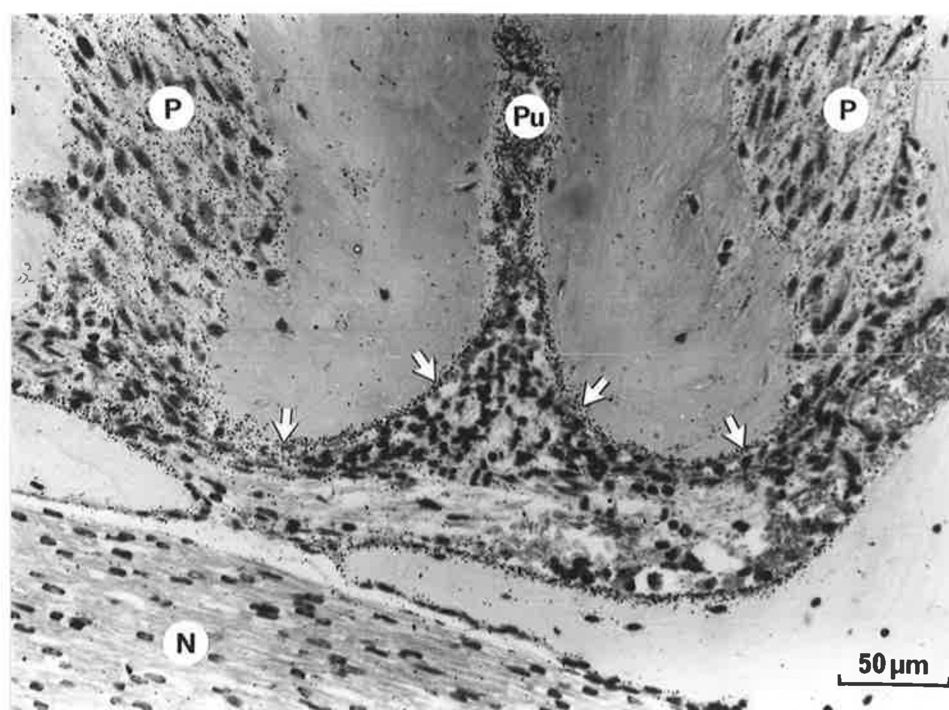

Fig. 15

The same band of silver grains is seen on the horizontal base of cellular cementum in this 42 day old mouse as in Fig. 14, extending into the root canal, arrowed. The band of silver grains does not extend out over the lateral surface of cellular cementum to which periodontal fibres are attached. P, periodontal ligament; Pu, pulp; N, inferior alveolar nerve. Nuclear fast red, indigocarmine and picric acid. x200

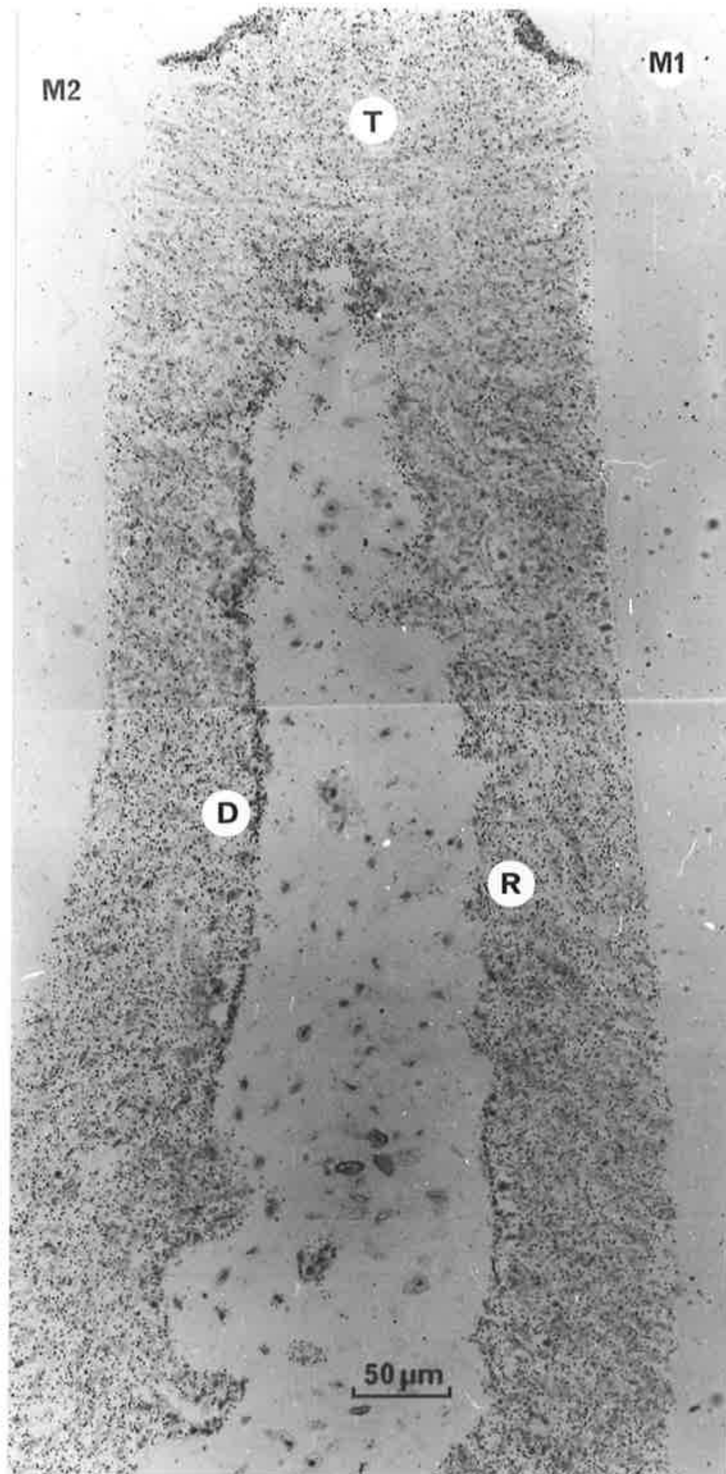

Fig. 16

Composite photomicrograph from an autoradiograph of a 42 day old mouse killed 4 hours after labelling. The interseptal bone has a depository surface adjacent to the mesial root of the second molar and a resorptive surface adjacent to the first molar root, a pattern consistent with distal drift of the teeth. Sharpey fibres show no autoradiographic evidence of turnover. M1, first molar; M2, second molar; T, transeptal fibres; R, resorptive surface; D, depository surface. Toluidine blue. x200

root surfaces. The majority of the activity associated with cellular cementum was on the apical face, extending from the root canal laterally to the oblique fibres of the periodontal ligament.

Alveolar Bone

A similar picture to that described for 23 day old mice was seen in the 42 day old group. Areas of increased grain density were found on both endosteal and periodontal surfaces (Fig. 16). Zones of endosteal apposition beneath areas of periodontal resorption were again seen (Fig. 17). On periodontal surfaces, however, although patches of increased grain density were seen on both mesial and distal surfaces, the majority of such areas were on the distal surfaces of the bony septa (Figs. 11, 16, 18).

Significant concentrations of grains were only seen marking bone surfaces. A few silver grains were seen over osteocyte lacunae in the older animals, mainly in the crestal regions of the interdental septa. Those regions of bone which incorporate transalveolar fibres were again unmarked by silver grains in autoradiographs (Figs. 16, 18).

C. *42 DAY OLD MICE KILLED AFTER 5 OR 9 DAYS (APPENDIX IV)*

Periodontal Ligament

Silver grains were present over principal periodontal fibres (Fig. 8). The concentration at 5 or 9 days was decreased compared to those animals killed after 4 hours. Quantitative grain counts were not assessed as the number of animals used was too small. The statistical limitation imposed by the small sample was demonstrated

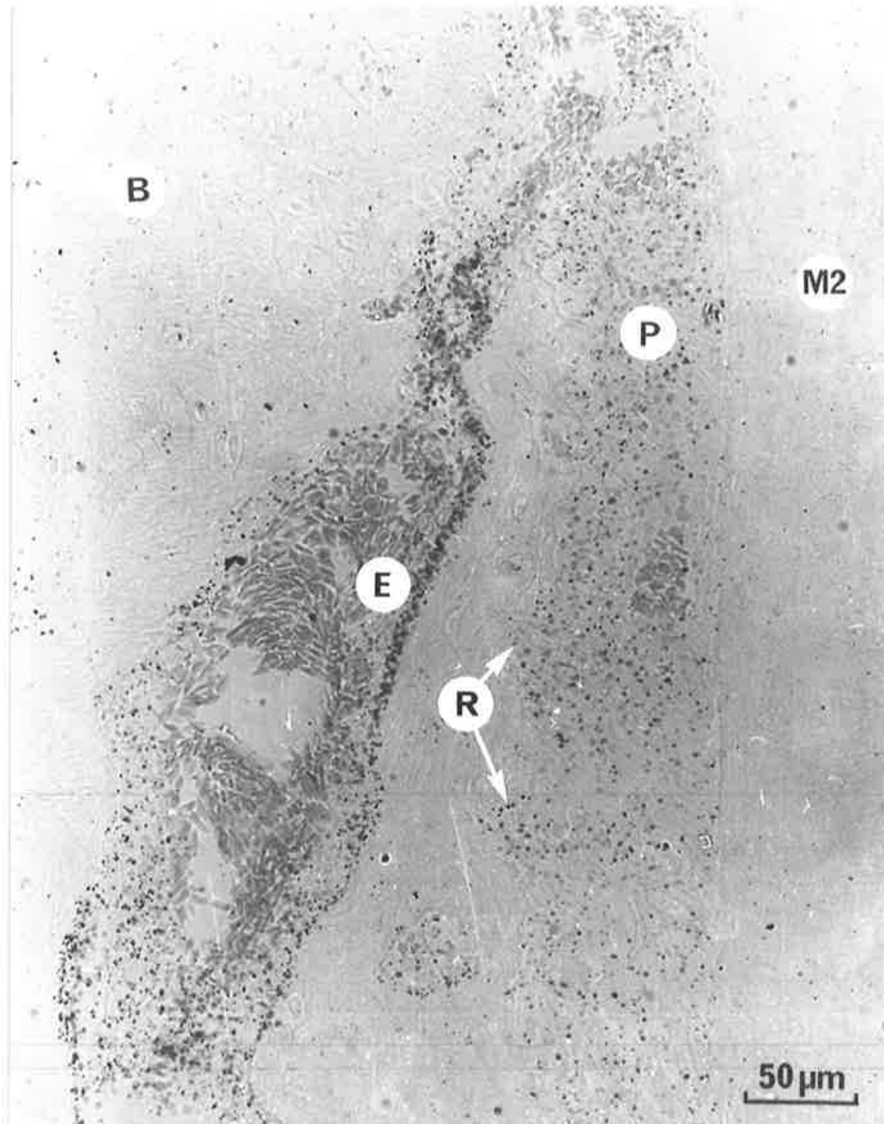

Fig. 17

Autoradiograph of a 42 day old mouse mandible showing endosteal apposition of bone in advance of a resorbing periodontal surface on the distal of the mesial root of the second molar. M2, second molar, distal root; B, bone; E, endosteal apposition; R, resorption lacunae; P, periodontal ligament. Unstained. x312

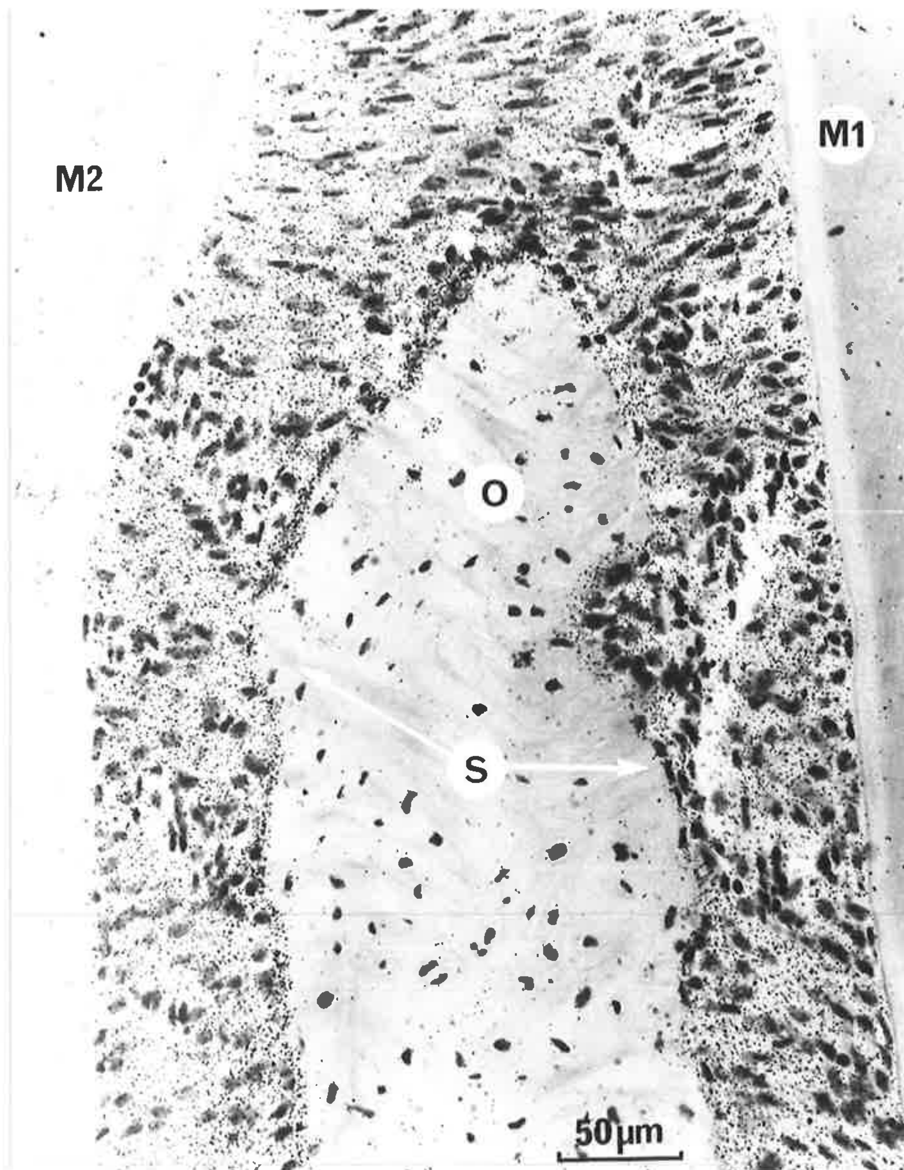

Fig. 18

Autoradiograph of a 42 day old mouse killed 4 hours after labelling. Silver grains mark the depository distal bone margin adjacent to the second molar. The few silver grains over the bone matrix are associated with osteocytes and not with the Sharpey fibres.
M1, first molar; M2, second molar; S, Sharpey fibres; O, osteocyte.
Nuclear fast red, indigocarmine and picric acid. x200

in animals killed at 5 or 9 days. The animal killed at 5 days (Fig. 19) seemed to have fewer silver grains over the periodontal ligament compared to the animal killed after 9 days (Fig. 20). Processing was the same for each of the autoradiographs.

Cementum and Dentine

The periodontal surface of cementum and the pulpal surface of dentine appeared similar, with none of the areas of high silver grain concentration which were seen in the animals killed after 4 hours, being present.

A line of silver grains was observed beneath the pulpal margin of the dentine in some areas. The line appeared over different areas in different teeth and was generally parallel to the surface. It seemed to delineate patches of newly formed dentine which looked like inverted saucers. Areas of apposition were seen most frequently on the floor of the pulp chamber, in the pulp horns or on the walls of the root canal. The line was generally deeper in the dentine in the animal killed 9 days after labelling.

In the apical region, a similar line of silver grains appeared in the cellular cementum. It was continuous with the line in the root canal beneath dentine. Peripherally it extended only to the sides of the root where the oblique fibres of the periodontal ligament were attached, and did not continue upwards beneath the entire outer surface of the cellular cementum (Fig. 12). Lines of silver grains were not observed over the matrix of acellular cementum. The bulk of cellular cementum deposition was associated with vertical root

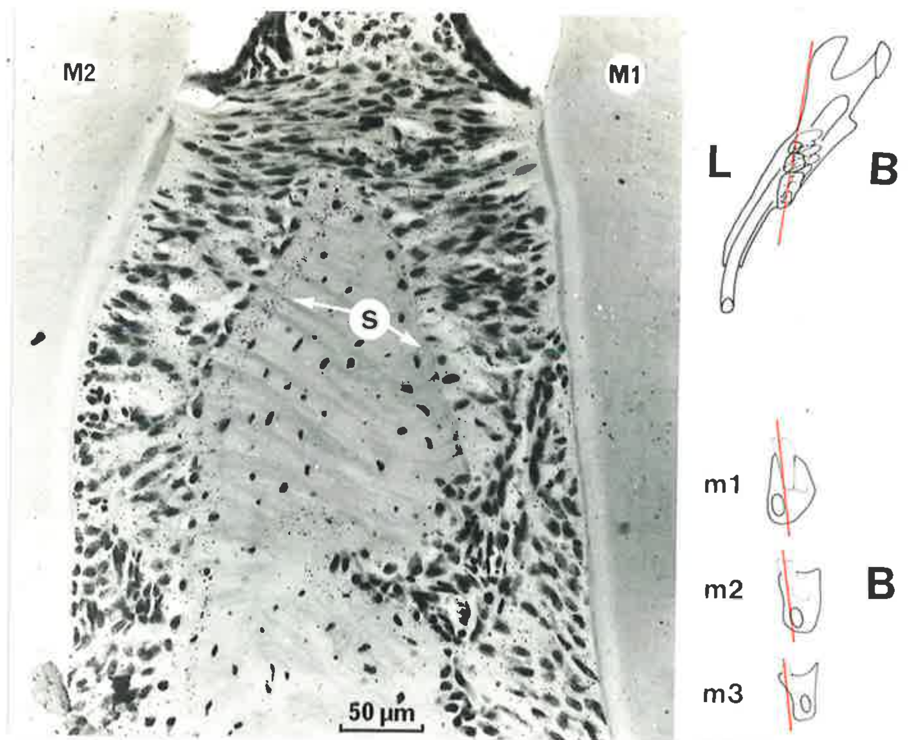

Fig. 19

Autoradiograph of an animal killed 5 days after labelling. The line of silver grains over the bone delineates bone deposition adjacent to the second molar. The concentration of silver grains is less than in similar areas in the animal killed after 9 days (Fig. 20). M1, first molar; M2, second molar; S, Sharpey fibres in bone. Nuclear fast red, indigocarmine and picric acid. x200

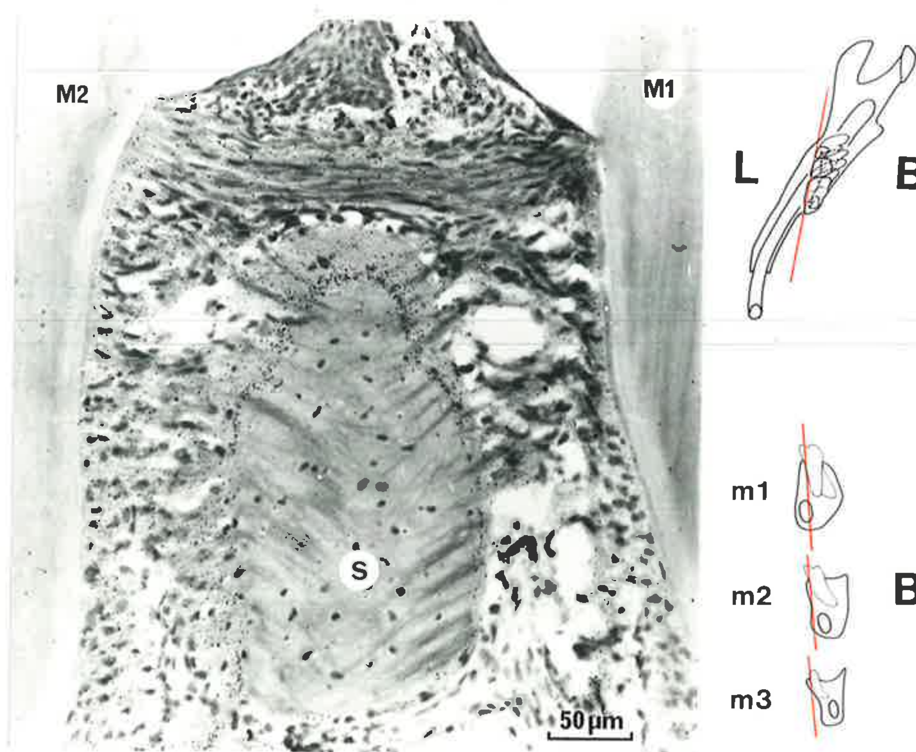

Fig. 20

Autoradiograph of an animal killed 9 days after labelling. The section has been taken from the lingual side. Note the V-shaped Sharpey fibre patterns and the extension of the line of silver grains on both the mesial and distal sides of the interseptal bone. The band is nearly twice as far from the surface compared with the animal killed after 5 days (Fig. 19). M1, first molar; M2, second molar; S, Sharpey fibres in bone. Nuclear fast red, indigocarmine and picric acid. x200

development. The lateral borders of subsequent layers of cellular cementum formed an extension of the tooth root to which periodontal fibres became attached.

Alveolar Bone

Due to the long delay between injection and sacrifice, the forming bone surfaces at the time of death did not incorporate significant amounts of radioactive proline. The bone surfaces in these animals therefore did not have the areas of increased silver grain concentration seen in the animals killed 4 hours after labelling.

The bone matrix had some quite different characteristics in these animals. Where Sharpey fibres were seen incorporated into the bone, a band of silver grains was routinely seen at some distance from the surface (Fig. 12). In the crestal areas, the band was approximately 40 μm from the bone surface in the animal killed at 9 days and approximately 20 μm in the animal killed at 5 days. A similar daily rate of bone formation of 4-4.5 μm was found in this area in both animals. The band was seen on the distal side of the interseptal and interradicular bone extending over the crest to the mesial side in some areas (Fig. 20). Patches of bone apposition were also seen in other areas. An explanation of the different views will be given in the discussion (Chapter 6).

Between the band and the depository surface, there was a much lower concentration of silver grains. In areas of bone beneath the band there were no silver grains above normal background levels, except around some osteocyte lacunae and over the marrow spaces

(Figs. 19, 20). The heavy line of silver grains was seen to be intermittent over Sharpey fibres. Towards the depository surfaces, the silver grains could be seen predominantly over the Sharpey fibres. The majority of silver grains over Sharpey fibres between the band and the depository surface, were incorporated into these fibres at the time of labelling. At this time the fibres were in the periodontal ligament. The activity was therefore associated with periodontal ligament fibres. Radioactive proline was not incorporated into Sharpey fibres in bone. Few silver grains were seen over bone matrix formed subsequent to that associated with the initial injection of radioactive proline (Figs. 7, 8).

In the lamellar bone beneath the apices of the teeth, a band of silver grains was also seen. No Sharpey fibres were incorporated in this area. The width of deposition of bone and cementum at the apex was similar, and corresponded in total to the width of bone deposited at the crestal areas (Fig. 12). The band of silver grains was seen in similar areas in the animals killed 5 or 9 days after labelling. However, after 9 days, the band was nearly twice as far from the surface as after 5 days in corresponding areas. It was noted that the concentration of silver grains in the band of the animal killed after 5 days, was less than that of the animal killed after 9 days.

At the alveolar crest, new fibre insertions had been formed since the time of labelling. These appeared to be clearly associated with only one tooth and were not formed by the incorporation of transeptal fibres (Fig. 8).

OBSERVATIONS OF SHARPEY FIBRE DEVELOPMENT

The development of the molar segment in white male mice was examined in animals from 10 days of age to adulthood. The observations were in close agreement with the reports of Cohn (1957) and Atkinson (1972). The sequence of eruption of the teeth and development of fibres of the periodontium is summarized in Table I.

TABLE I

CHRONOLOGY OF DEVELOPMENT OF THE MANDIBULAR MOLARS AND PERIODONTAL STRUCTURES

	1st Molar	2nd Molar	3rd Molar
Crown completed	<10 days	10 days	18 days
One third root length	<14 days	14 days	20 days
Two thirds root length	16 days	18 days	23 days
Root full length	20 days	23 days	28-42 days
Eruption	16-18 days	18 days	23-28 days
Sharpey fibres in cement	10 days	14 days	20 days
Sharpey fibres in bone	14-16 days	16 days	23 days

The 10 day old mice had all 3 mandibular molars unerupted in the jaws, although in varying stages of development. The first and second molars did not have bone overlying the crown, whereas the third molar was still in a bony crypt (Fig. 21). The fibrocellular investing layer (Ten Cate, 1969) was observed immediately surrounding each developing tooth.

The investing layer formed a fine capsule with the fibrous stroma parallel to the outer surface of the tooth. Over the crowns the layer followed the smooth convex outer surface of external enamel epithelium but was not closely adapted to the cusps. Between the teeth, above the developing interseptal bone, the adjacent investing layers merged.

The first molar had less than one third of its root formed. Fibroblasts and extracellular material were orientated in a superior oblique direction from the root surface, blending with the investing layer over the crown (Fig. 22). There was close approximation of extracellular material to the outer surface of the dentine. Morphologically extracellular material was attached to the root via cementum. Histological stains did not clearly delineate either collagen fibres or cementum in the 10 day old mice (Fig. 22).

Apical growth of the roots was associated with movement in the teeth in an occlusal direction. Concurrently, trabeculae of bone extended occlusally maintaining a level somewhat coronal to the cemento-enamel junction and forming the outer limit of the periodontal space. Osteoblasts on the surface of the trabeculae laid down the matrix of the bone. Some osteoblasts were incorporated into the matrix as osteocytes. New spicules of bone were developed on the first formed bone. The intrinsic fibres of the trabeculae were arranged parallel to the long axis of each spicule, and in the general direction of the connective tissue stroma of the region. No periodontal fibres were observed attached to bone in 10 day old mice (Fig. 22).

At 14 days of age the roots of the first and second molars had elongated. The first molar roots were half formed and those of the

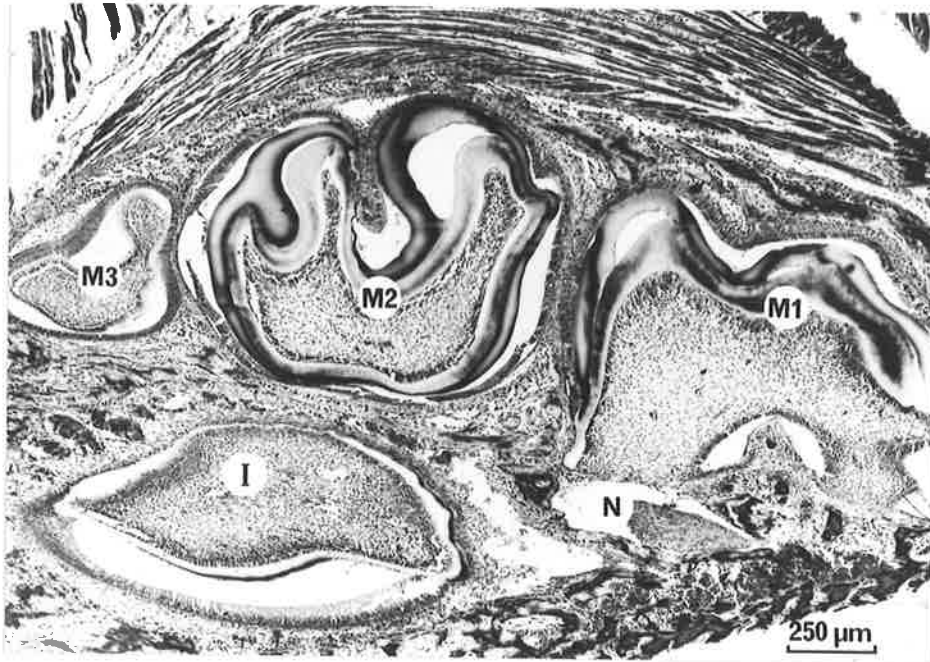

Fig. 21

Developing dentition in the mandible of a 10 day old mouse. The section passes through the roots of the first molar and the crowns of the second and third molars. M1, first molar; M2, second molar; M3, third molar; I, incisor; N, inferior alveolar nerve. Pollack's trichrome. x40

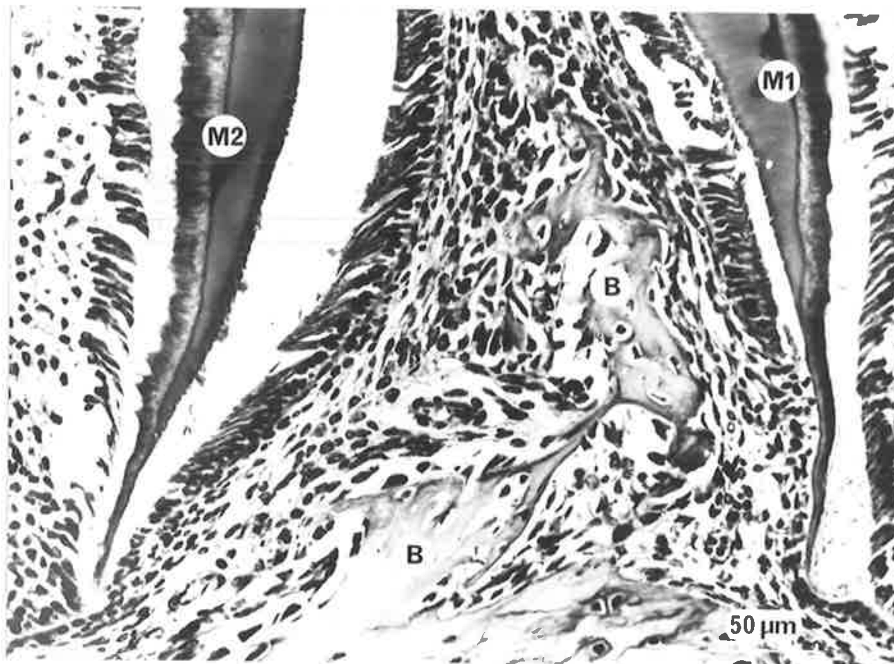

Fig. 22

Trabeculae of bone forming the interdental septum in a 10 day old mouse. Cells and extracellular material are in intimate contact with the distal root surface of the first molar. No attachment of Sharpey fibres to bone can be seen. M1, first molar; M2, second molar; B, interseptal bone. Pollack's trichrome. x200

second molar approximately one third. The investing layer over the crowns was thicker and more fibrous. Beneath the forming roots the investing layer was still evident. In the region between the distal root of the first molar and the bone, the tissue was more fibrous and both the cells and extracellular material were predominantly orientated in a superior oblique direction, from more apical on the root to the alveolar crest of bone (Fig. 23).

The tissue in relation to the second molar was less well organised, being similar to that of the first molar at 10 days. Fibres could be seen attached to the roots of both molars, although cementum was not histologically evident.

The extracellular material, orientated in the superior oblique direction from the first molar root to the alveolar bone, appeared to be in intimate contact with the bone (Fig. 23). However, it was not possible to determine whether attachment of fibres to bone had occurred, from the histologic examination of the material. The trabeculae forming the interseptal and interradicular bone were more extensive at 14 days, with their intrinsic fibres parallel to the surface of each spicule. In deeper areas, the trabeculae were thicker and endosteal apposition was forming the inner layer of primary osteones. No evidence of major remodelling of the first formed bone was seen.

At 16 days there was a considerable advancement in the development of the structures. The first molar root was approximately two thirds formed and that of the second molar one half. The third molar was in the bell stage, totally within a bony crypt. and with two thirds of the

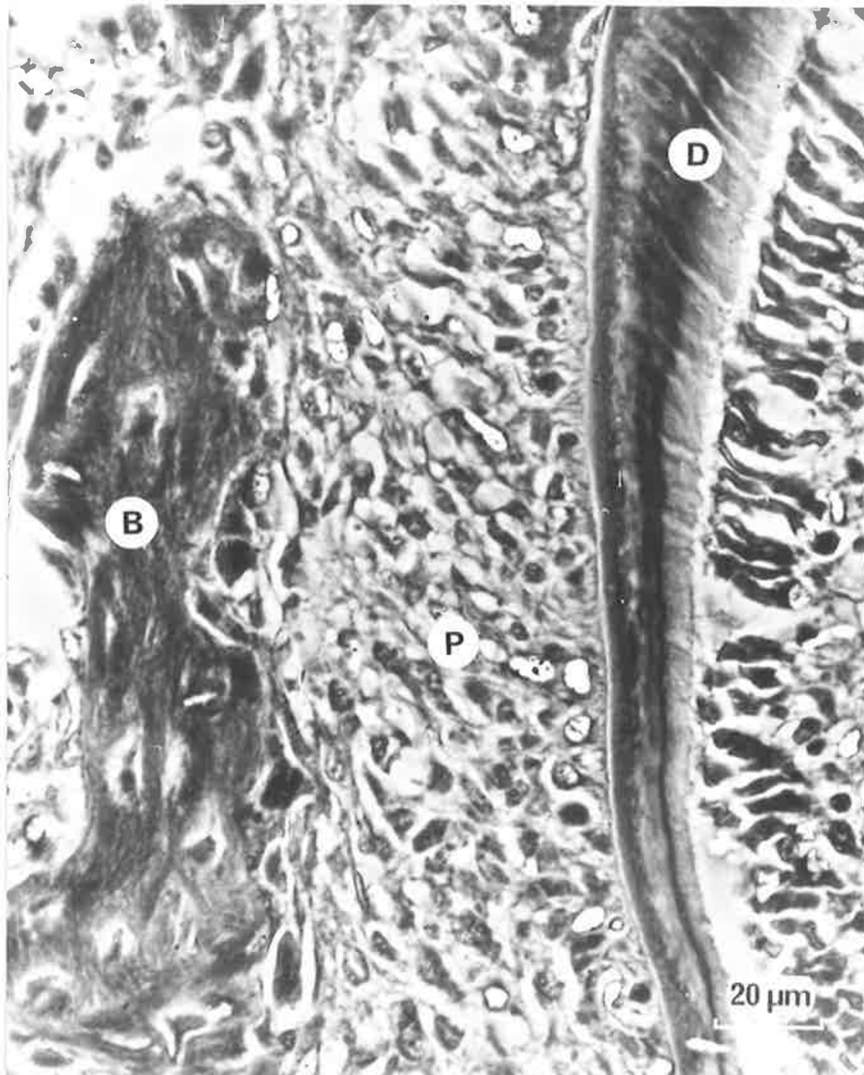

Fig. 23

Distal side of the distal root of the first mandibular molar of a 14 day old mouse. Organised fibre bundles in the periodontal ligament are not evident. Extracellular material in the ligament space is attached to the root and closely approximates the bone. D, dentine; B, bone; P, periodontal ligament. Phase contrast, Pollack's trichrome. x500

crown formed. Both first and second molars were covered by oral epithelium and the persisting investing layer (Fig. 24). Distinct fine fibre bundles were seen in the periodontal ligament.

The alveolar bone forming in relation to the teeth, extended above the level of the cemento-enamel junction mesially and distally of both first and second molars. The teeth were unerupted and periodontal fibres were aligned in a superior oblique direction from the forming apex to the alveolar crest. Transeptal fibres were evident at this age arcing over the alveolar crest.

Sharpey fibres were inserted into the cementum of the first and second molars. Fine Sharpey fibres were also inserted into the alveolar bone in relation to both the first and second molar teeth (Fig. 25).

In the 18 day old mice both the first and second molars had erupted. The first molar had more than two thirds of its root formed, whilst the second molar had between one half and two thirds. The third molar crown was nearly complete (Fig. 26).

The first and second molars now had a different relation to the supporting bone with the cemento-enamel junction occlusal to the alveolar crest. Periodontal fibres were larger, more clearly defined and orientated in the same direction as in the functional teeth in the adult. No investing layer remained over the crown and the transeptal fibre system was further developed. On the distal of the second molar, the investing layer of the third molar was seen, in part, to be continuous with some fibres attaching to the root of the

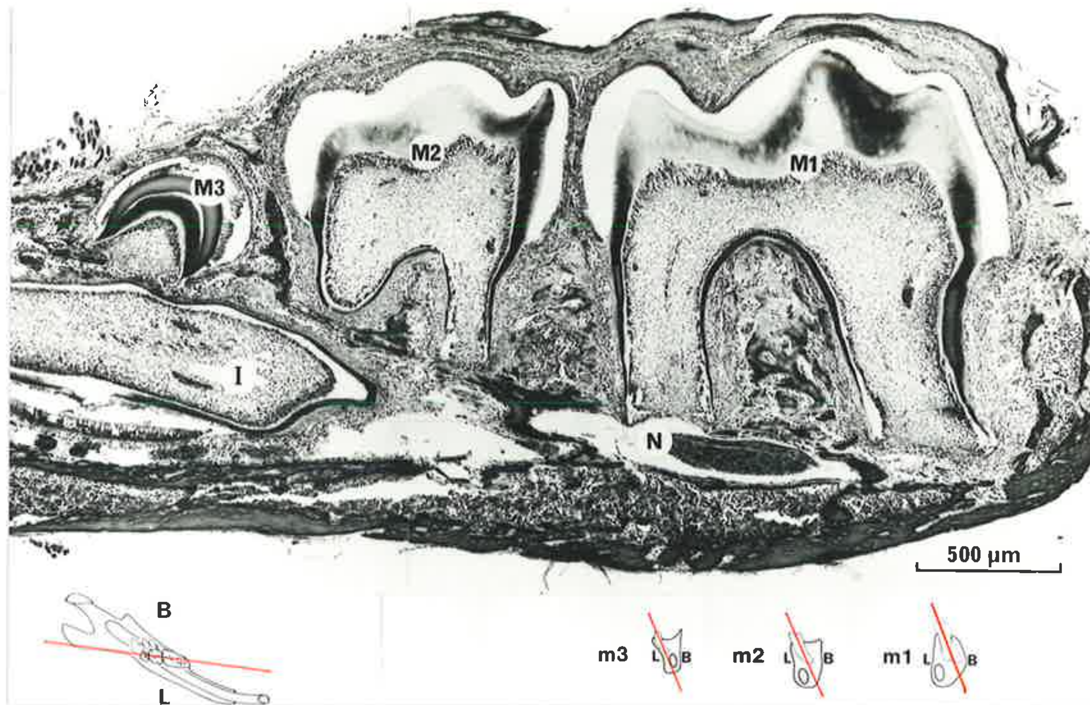

Fig. 24

Mesiodistal section showing the unerupted mandibular molars in a 16 day old mouse. The investing layer persists over the crowns and has developed into the periodontal ligament between the bone and the tooth roots. M1, first molar; M2, second molar; M3, third molar; I, incisor; N, inferior alveolar nerve. Pollack's trichrome. x40

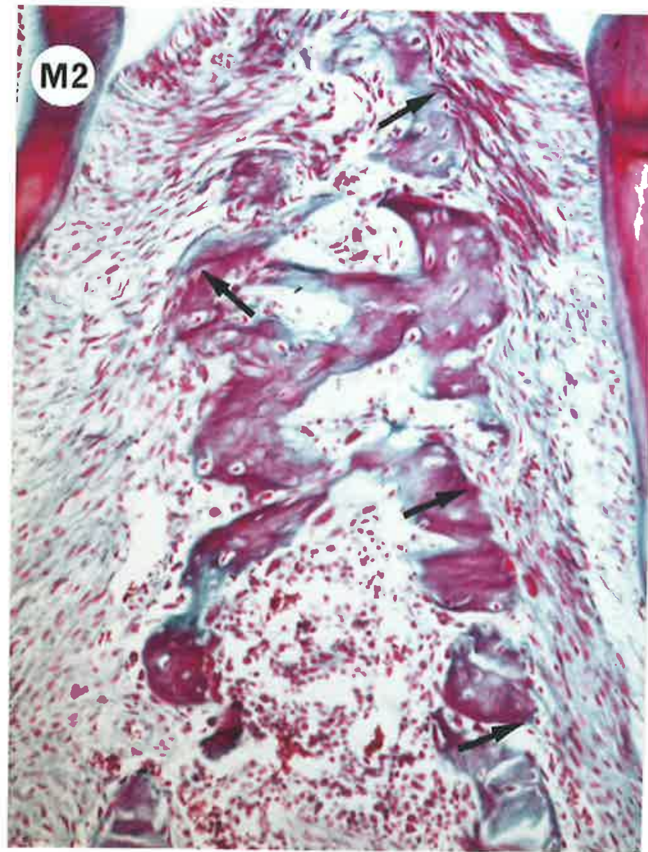

Fig. 25

Interproximal area between the mandibular first and second molars of a 16 day old mouse. Fine Sharpey fibres, similar in size to the periodontal fibres, can be seen attached to alveolar bone, arrowed. M2, second molar. Pollack's trichrome. x40

second molar near the cemento-enamel junction (Fig. 26). The periodontal fibres at the bone surface appeared thicker than the fine fibres which were first observed at 16 days (Fig. 26).

In the interradicular area, oblique fibre groups were attached to the root and alveolar bone. The interradicular crest was however formed of trabeculae which did not incorporate Sharpey fibres. The organised alveolar crest and horizontal fibre groups which were seen in the interseptal area were not formed interradicularly (Figs. 27, 28). At the interseptal crest, trabeculae were no longer formed. The increase in height of the crest was by surface bone deposition which surrounded the attached Sharpey fibres (Fig. 28). New Sharpey fibres were also incorporated as part of the bone matrix at the alveolar crest, directed along the line of the alveolar crest fibres.

At 20 days the first molar roots were nearly completed in length although the dentine was still very thin. The second molar had more than two thirds of the root formed. Eruption had proceeded with the growth of the jaws. The apices of the teeth throughout development maintained a close association to the inferior alveolar nerve. Eruption relative to the nerve and basal bone was accomplished by apical root growth and bone apposition at specific sites on the socket surface. Bone was resorbed from other areas to maintain the normal relation of tooth to bone. New periodontal fibres formed apically, associated with Sharpey fibres attaching to the lengthening tooth root. New periodontal fibres also formed coronally associated with bone growth at the alveolar crest (Fig. 29).

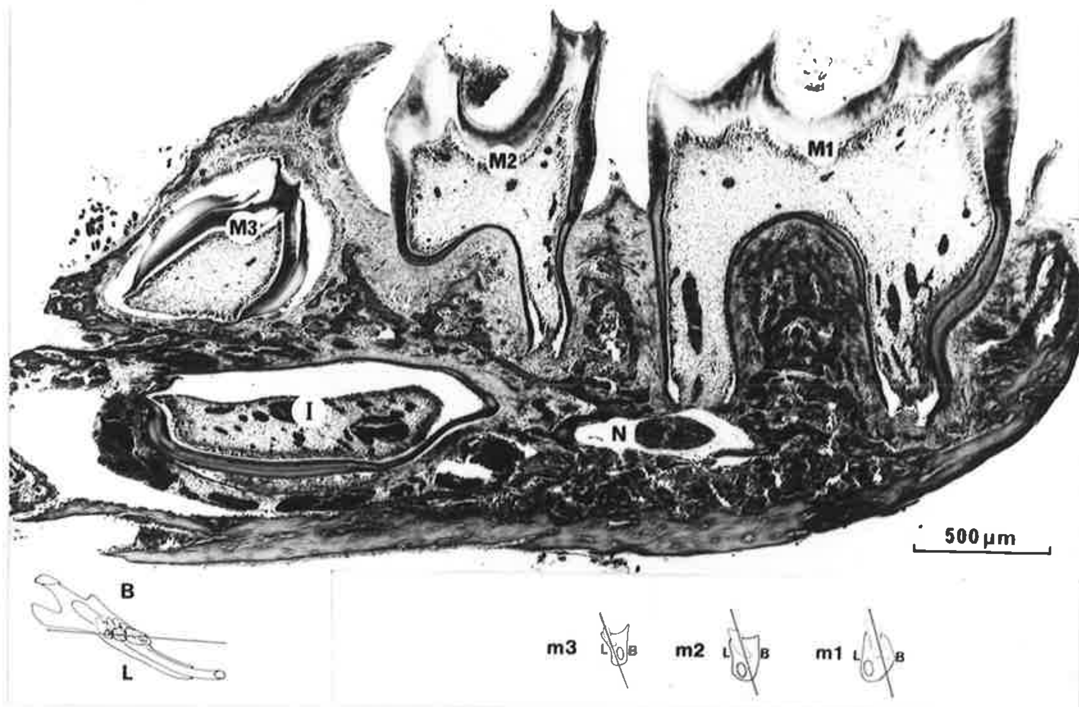

Fig. 26

Mesiodistal section from an 18 day old mouse mandible. The apex of the first molar root has maintained a similar relation to the inferior alveolar nerve as at 16 days. The third molar is surrounded by the investing layer which blends with the periodontal ligament of the second molar. M1, first molar; M2, second molar; M3, third molar; I, incisor; N, inferior alveolar nerve. Pollack's trichrome. x40

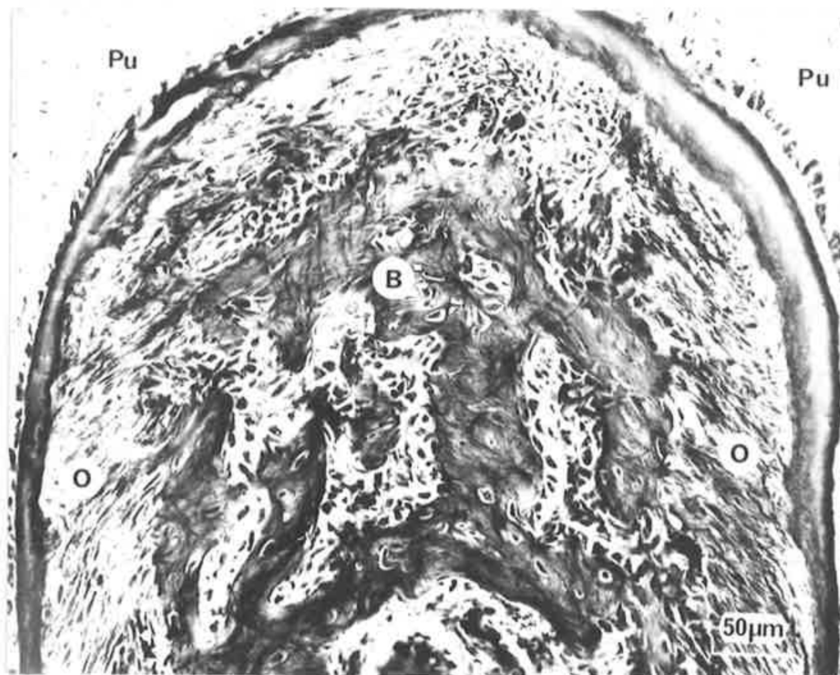

Fig. 27

Interradicular crest beneath the first mandibular molar of an 18 day old mouse. Oblique fibre groups are organised on each root surface. However the crestal tissue is associated with proliferating trabeculae of bone. Pu, pulp; B, bone; O, oblique periodontal fibres. Pollack's trichrome. x200

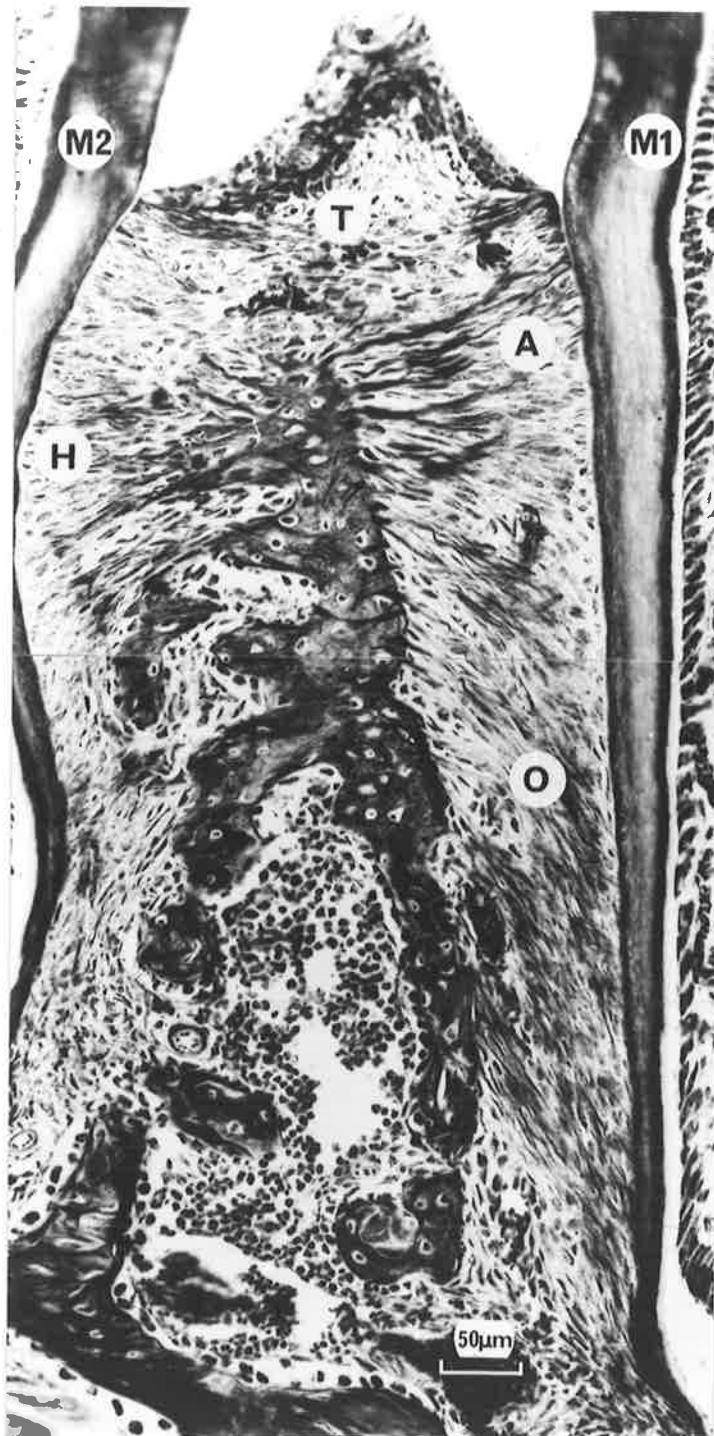

Fig. 28

Interseptal bone between mandibular first and second molars of an 18 day old mouse. Periodontal fibres have become thicker and are now arranged as in the adult periodontal ligament. M1, first molar; M2, second molar; T, transeptal fibres; A, alveolar crest group; H, horizontal fibre group; O, oblique fibre group. Pollack's trichrome. x200

Between the first and second molars, development associated with each of the teeth proceeded concomitantly. The forming crest incorporated, as Sharpey fibres, alveolar crest fibres from the periodontal ligaments of each of the first and second molars as an integral part of the matrix. These Sharpey fibres were orientated parallel to alveolar crest fibres from each of the teeth and formed an intermingling V-shaped pattern in the bone. There was no demarcation line in the bone between the fibres from each tooth (Figs. 3, 28, 29). At this stage no Sharpey fibres could be traced in serial sections across the interseptal bone.

The third molar periodontium developed in an essentially similar manner but was later chronologically. At 23 days approximately two thirds of the root was formed, with fibres attached to both cementum and bone (Figs. 30, 31). To the mesial, the bone associated with the distal root of the second molar was already present. Lamellar bone which incorporated fine Sharpey fibres from the third molar periodontium was laid down on the bone distal to the second molar. A distinct reversal line was seen separating this more recently formed bone from that of the second molar (Figs. 30, 31). The reversal line stained strongly with Pollack's trichrome (Fig. 31) and aldehyde fuchsin, both mucopolysaccharide stains, but not at all with silver stain (Fig. 30).

The first Sharpey fibres related to the third molar were retained in the bone of the 28 day old mice (Fig. 32). Sharpey fibres in bone were generally of similar size except in the early developmental stages when fine fibres were attached. At such early stages there

Fig. 29

The interseptal bone crest of a 20 day old mouse incorporates newly formed Sharpey fibres as part of the matrix. New periodontal fibres also form at the developing apex of the tooth. M1, first molar; M2, second molar. Pollack's trichrome. x200

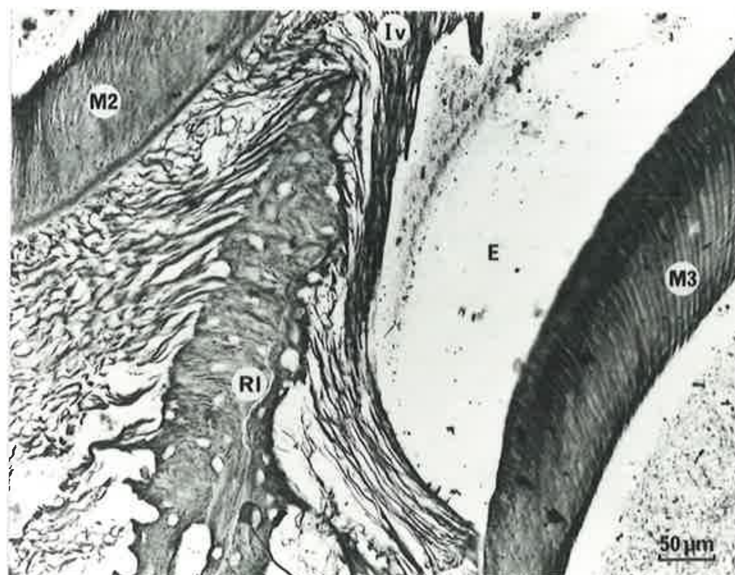

Fig. 30

Initial attachment of periodontal fibres from the third molar periodontium to bone in a 23 day old mouse. A distinct reversal line separates the Sharpey fibre bone of the second molar from that of the third molar. M2, second molar; M3, third molar; Iv, investing layer; E, enamel space; Rl, reversal line. Gordon and Sweet silver impregnation and light green. x250

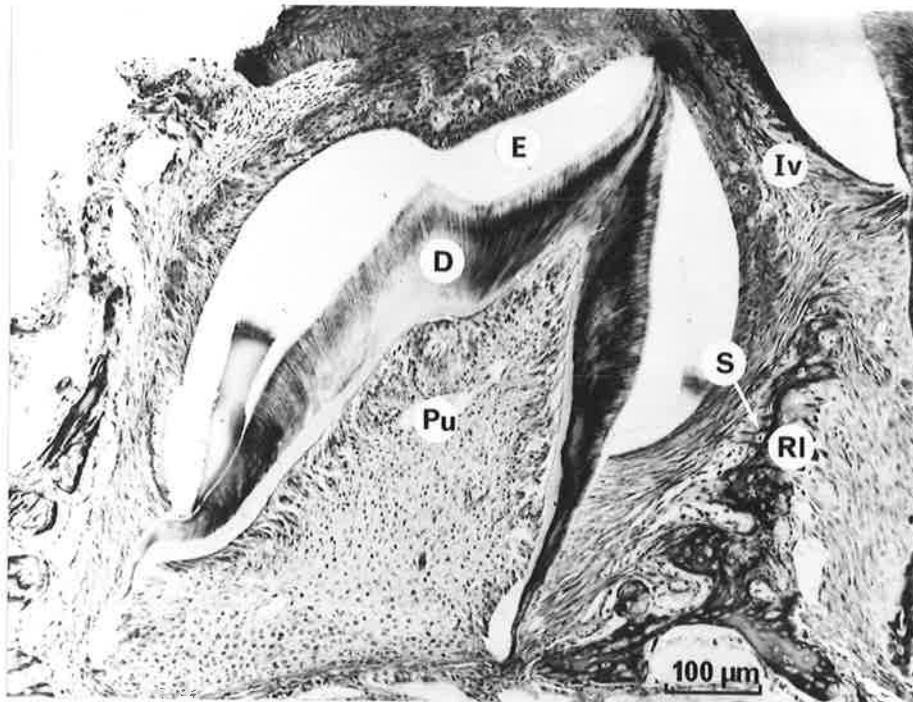

Fig. 31

Periodontal fibres of the developing third molar in a 23 day old mouse become attached to bone independently from the periodontal fibres of the second molar. E, enamel space; D, dentine; Pu, pulp; Iv, investing layer; S, Sharpey fibres associated with the third molar; Rl, reversal line. Pollack's trichrome. x100

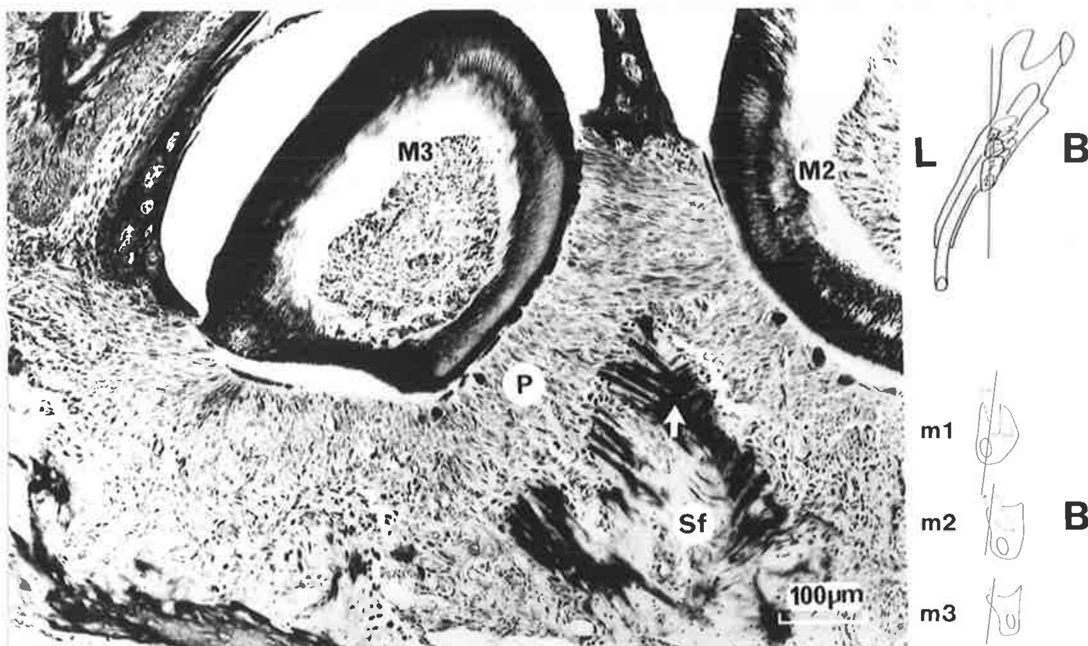

Fig. 32

Mesiodistal section from the lingual side of the third molar of a 28 day old mouse. Note the V-shaped junctions of Sharpey fibres from the second and third molars, arrowed. M2, second molar; M3, third molar; P, periodontal fibres; Sf, fine Sharpey fibres, the preserved first attachments as seen in Fig. 31. Pollack's trichrome. x100

were no major periodontal fibre bundles to be seen attached to the bone (Figs. 25, 31).

SHARPEY FIBRE PATTERNS WITHIN BONE

The first Sharpey fibre insertions were seen at 16 days associated with the unerupted first and second molars. They were aligned, as the periodontal fibres, in a superior oblique direction from the root occlusally into the crests of bone. The major part of the interdental bone at this stage was trabeculated with large marrow spaces and without deep Sharpey fibre insertions (Fig. 25).

By 18 days, after eruption of the first and second molar teeth, there was a rearrangement of the periodontal ligament to include alveolar crest and horizontal fibre groups. Sharpey fibre insertions were formed in the direction of, and continuous with, these new fibre groups. At the alveolar crest Sharpey fibres formed V-shaped junctions in the bone and extended superiorly as alveolar crest fibres towards the cemento-enamel junction of each adjacent tooth (Fig. 28).

At 20 days the pattern was similar with continuing apposition of bone at the crestal areas incorporating newly formed, alveolar crest fibres. The interradicular bone increased in height and bulk by development of trabeculae. The vertical surfaces of the interradicular bone incorporated oblique periodontal fibres as Sharpey fibres. Periodontal fibres appeared to be arranged so as to resist intrusive and lateral loading. Consequently, few Sharpey fibres were seen in the furcation areas (Fig. 27).

At 23 days, with eruption of the first and second molars to the level of the occlusal plane, the coronal half of the interseptal bone included many buried Sharpey fibres, with the associated intrinsic fibre matrix and a few vascular channels. The apical half had Sharpey fibres incorporated on its periodontal margins and a less ordered inner aspect, with varying degrees of compaction of the cancellous spaces. The original trabeculae of bone formed during development were still part of the bone matrix (Fig. 33).

Formation of new fibre attachments occurred at the crest of bone, generally in a direction towards the cemento-enamel junction. The formation of new alveolar crest fibres in the ligament, was associated with a reorientation of the more apically placed alveolar crest fibres to horizontal and oblique fibres. This change in orientation was recorded, either in the curved path of the fibres in bone, or as an angle between the Sharpey fibre and its periodontal extension (Fig. 33). Where this angle became too great, the periodontal fibre appeared to be severed and reattached by spot deposition of bone in its new functional direction in the ligament. New periodontal fibres also formed in the apical part of the ligament and became attached to the surface of the lengthening root.

In animals older than 23 days, Sharpey fibre insertions on the distal alveolar walls were deeper than those on the mesial. The Sharpey fibres which were inserted more deeply appeared to maintain their direction established in the bone (Fig. 34).

In the 70 day old animals Sharpey fibres were seen to extend continuously from the alveolar crest fibres of the distal tooth across

the interdental bone to the adjacent alveolo-periodontal interface (Fig. 35). However, examination of this area in the scanning electron microscope, clearly showed that these Sharpey fibres were not continuous with the fibres from the periodontal ligament of the mesial tooth (Fig. 36). The mesial facing alveolar surfaces had characteristically an eroded margin and no deep Sharpey fibre insertions (Figs. 35, 36). Osteoclasts were also occasionally seen.

Horizontal sections clearly showed evidence of the distal and buccal direction of drift of the teeth (Fig. 37). Lamellar bone with incorporated Sharpey fibres was seen to the mesial and lingual of each tooth root. In the interdental region between the first and second molar, the entire partition was formed of lamellar bone incorporating Sharpey fibres from the second molar. Towards the distolingual aspect of the septum, however, there were some Sharpey fibres associated with the first molar root (Fig. 37).

Buccolingual sections showed long Sharpey fibres continuous with periodontal fibres on the lingual aspect (Figs. 38, 39, 40). On the buccal aspect, where sections were from the distal side of a root (line X on Fig. 37), no long Sharpey fibres continuous with periodontal fibres were seen (Fig. 38). However, where sections passed through the mesial of a root (line Y on Fig. 37), Sharpey fibres continuous with periodontal fibres were seen both on the buccal and the lingual sides (Fig. 39). (See Chapter 6 for discussion.)

On the lingual aspect, near the crest, Sharpey fibres perforated the lingual cortical plate. However, they terminated flush with the

bone surface and were not seen to enter the periosteum. More apically a layer of periosteal bone covered the Sharpey fibre bone (Fig. 40). The path of Sharpey fibres in the lingual bone was not always straight. A gradual curve was observed in some fibres from a superior oblique direction to an inferior oblique direction, parallel to the oblique fibres of the periodontal ligament (Figs. 38, 39, 40). The change in orientation of Sharpey fibres in bone on the lingual side, was similar to the patterns described in the interseptal bone in mesiodistal sections (Fig. 33). However, the patterns were associated with a tooth root on only one side.

Fig. 35
ADULT x200

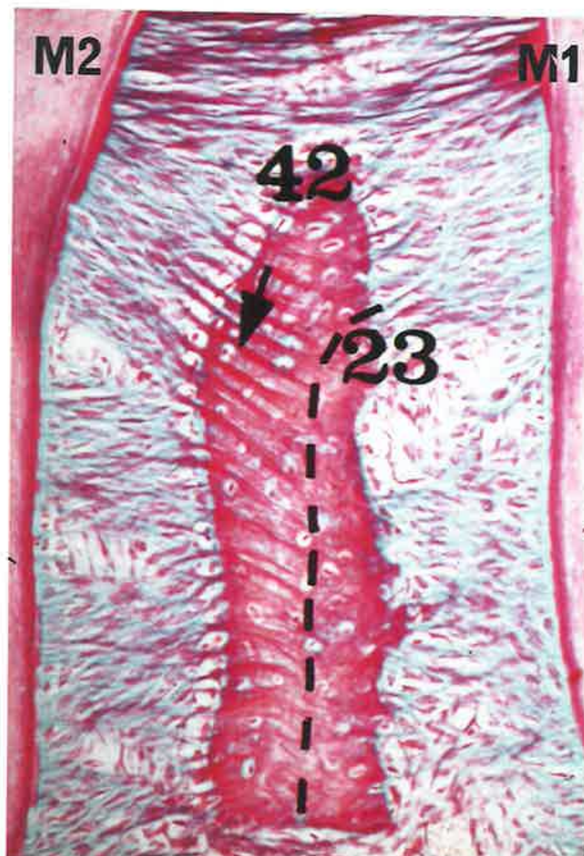

Fig. 34
42 DAY OLD x250

Fig. 33
23 DAY OLD x250

The sequence of photomicrographs of the interseptal bone between the first and second mandibular molars shows the development of patterns of Sharpey fibres in bone due to eruption, Fig. 33, and continuing distal, buccal and occlusal physiologic migration of the teeth (to the top and left of the page), Figs. 34 and 35. Curved fibre pathways, arrowed Fig. 33, show the reorientation of alveolar crest fibres to oblique fibres. Osteoclasts can be seen on the resorptive surface of Fig. 35, adjacent to the first molar. The numbers refer to the animal's age and the estimated position of the alveolar crest at that stage. M1, first molar; M2, second molar. Pollack's trichrome.

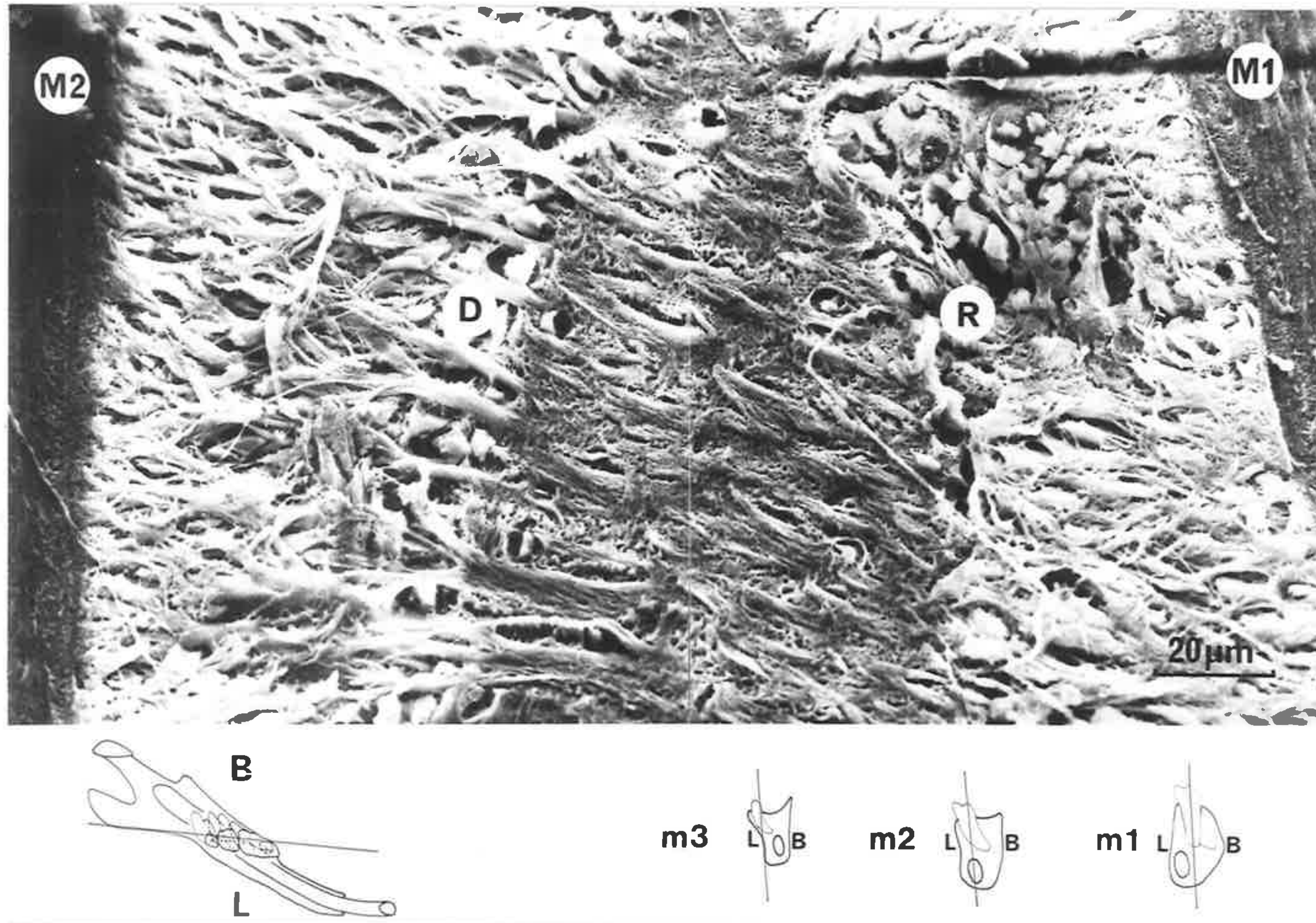

Fig. 36

Scanning electronmicrograph of the interseptal bone between the first and second mandibular molars of an adult mouse aged 70 days. The periodontal ligament on the depository surface has more large organised collagen fibre bundles which insert deeply as Sharpey fibres in the bone. Conversely, the fibres from the first molar are attached only to the surface of the bone. M1, first molar; M2, second molar; D, depository surface; R, resorptive surface. x450

Y SECTION Fig. 39

X SECTION Fig. 38

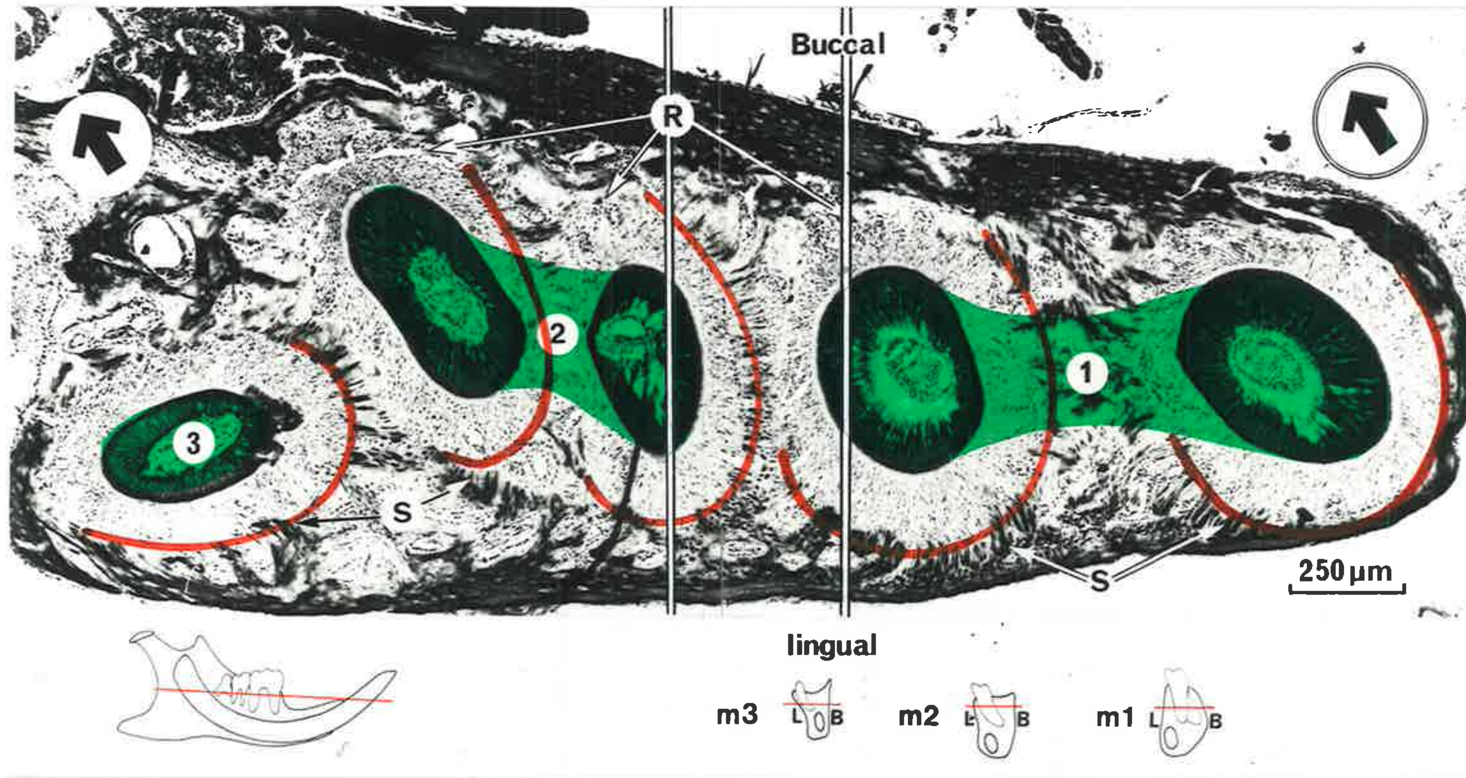

Fig. 37

Horizontal section of a 28 day old mouse. Fibre patterns indicate that the direction of drift is distally and buccally as marked by the large arrows. Sharpey fibres have been incorporated at depository surfaces, marked by the heavy red lines. The buccolingual section of Fig. 38 will have a depository surface only on the lingual side. Conversely the section of Fig. 39 will show a depository surface on both the buccal and lingual sides. 1, first molar; 2, second molar; 3, third molar; R, resorptive surfaces; S, Sharpey fibres. Pollack's trichrome. x60

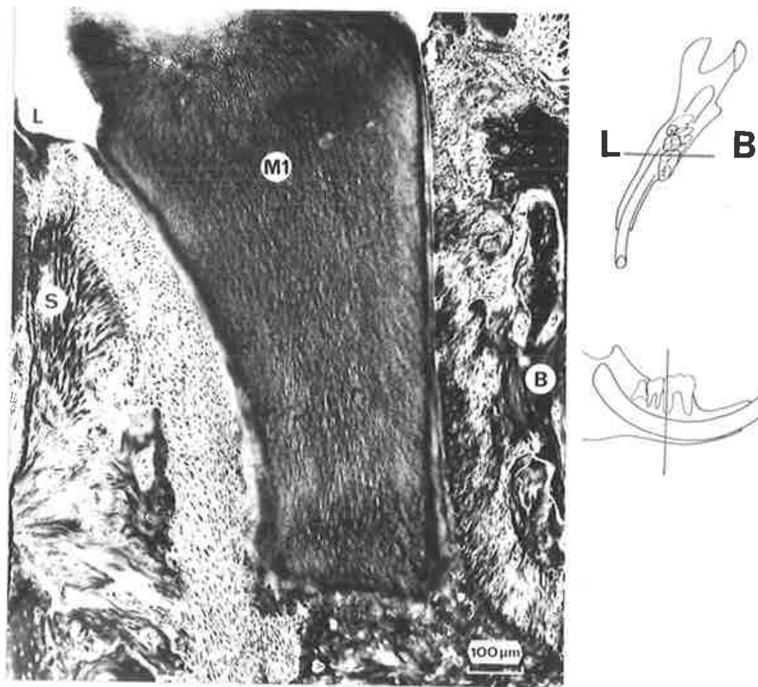

Fig. 38

Buccolingual section from an adult animal through the distal of the distal root of the first molar, line X on Fig. 37. The lingual side is depository and the buccal side is resorptive. M1, first molar; S, Sharpey fibres; L, lingual side; B, buccal side. Pollack's trichrome. x80

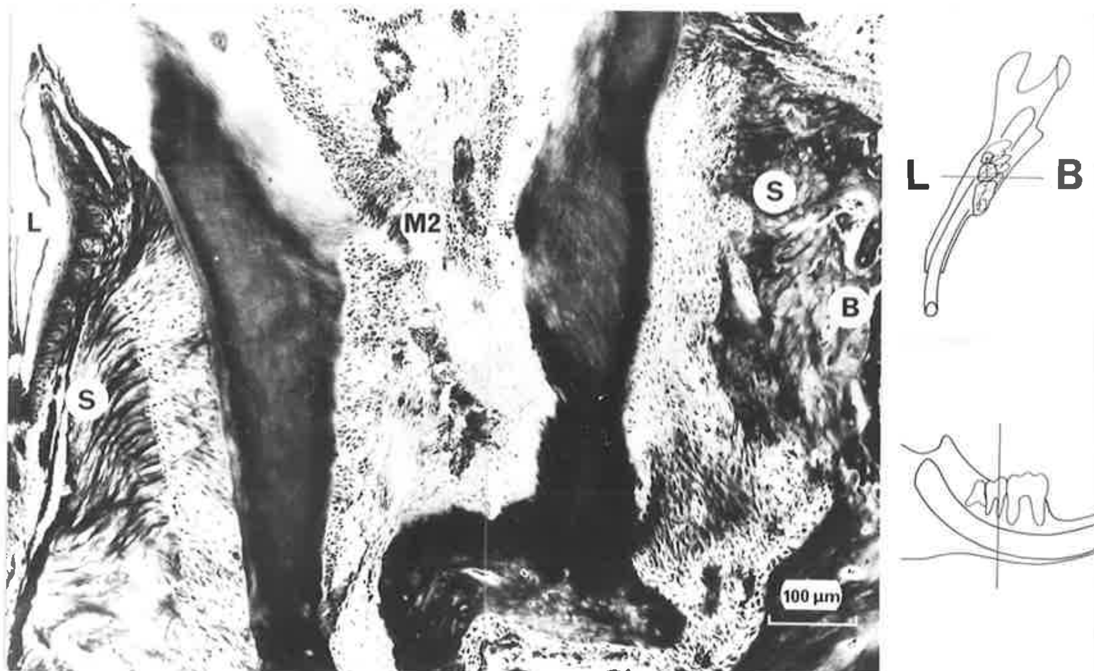

Fig. 39

Buccolingual section from the same animal as Fig. 38, through the mesial of the mesial root of the second molar, line Y on Fig. 37. Both the lingual and buccal sides are depository. M2, second molar; S, Sharpey fibres; B, buccal; L, lingual. Pollack's trichrome. x125

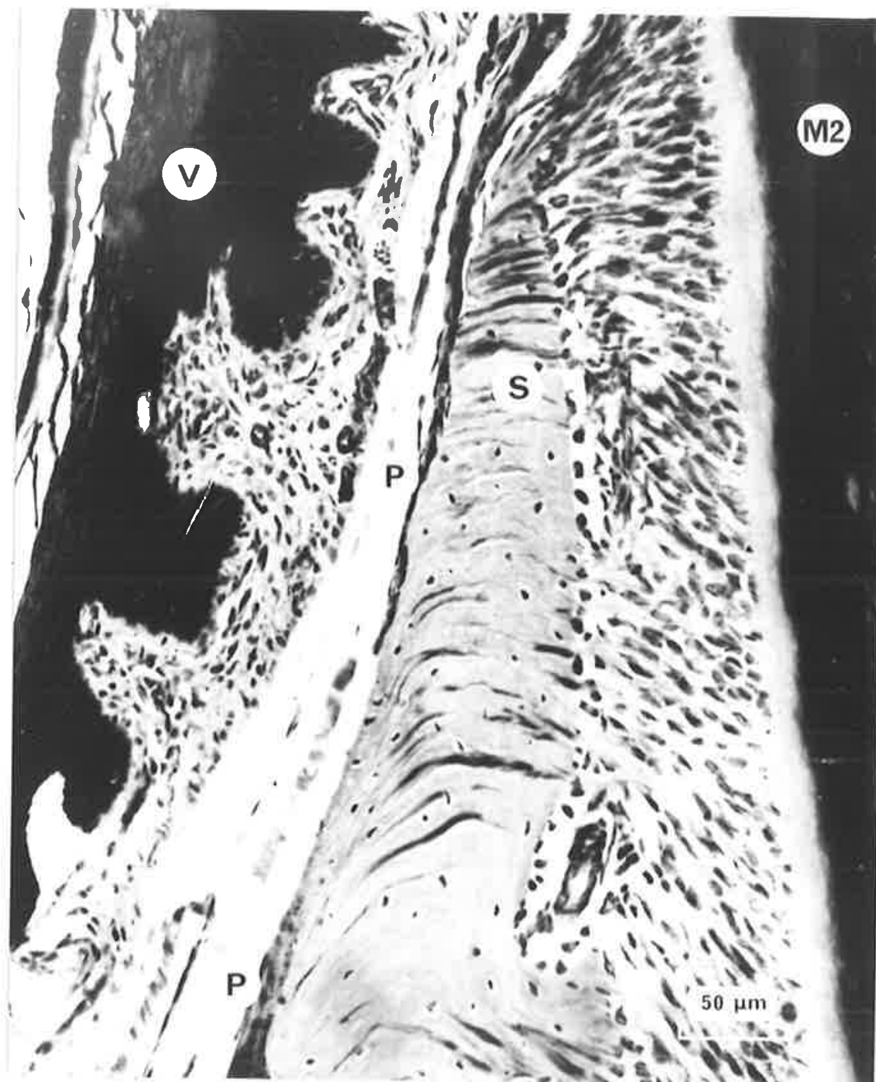

Fig. 40

Buccolingual section of an adult mouse showing the lingual cortical plate adjacent to the mesial root of the second molar. Sharpey fibres traverse the coronal part of the plate but do not enter the periosteum. Periosteal bone covers the deeper areas of Sharpey fibre bone. Curved fibre pathways analogous to those seen in the mesiodistal sections are seen in the bone. S, Sharpey fibres; P, periosteum and periosteal bone; M2, second molar; V, vestibular epithelium. Pollack's trichrome. x200

CHAPTER 6

DISCUSSION

AUTORADIOGRAPHY

Following labelling of experimental animals with radioactive proline, subsequent procedures yielded histologic sections overlaid with a photographic emulsion in which developed silver grains marked sites of radioactive emissions. Tritium is an unstable isotope of hydrogen, half life 12.3 years, which decays by emitting a β particle of maximum energy 18 kev (Rogers, 1973; Tonna, 1974). This is a low energy particle which is readily absorbed by adjacent tissues. Particles have virtually no chance of travelling further than 5 μm through tissue. Therefore sections 5 μm and greater can be considered of infinite thickness with no increase in the number of recorded decays being noted by increasing the tissue thickness.

The distance that tritium β particles can penetrate into nuclear emulsion is also important. For practical purposes, the maximum distance is 3 μm, a thickness which is exceeded by most liquid emulsion techniques (Rogers, 1973). The combination of low energy β particles and a moderately sensitive Ilford K2 nuclear emulsion provided high resolving power with small silver grains being developed very close to the points of decay. Exposure time of 35 days was found to produce a good concentration of silver grains over the periodontal ligament with minimal background radiation.

Previous investigations trace radioactive label through tissue compartments over time following intraperitoneal injection (Ross and Benditt, 1963; Carneiro and Fava-de-Moraes, 1965). Based on these earlier reports a time interval of 4 hours between injection and sacrifice was selected in this study. As anticipated high concentrations of label were observed over extracellular materials at this time. Furthermore, the distribution of label in the mice of this investigation was similar to that previously reported in mice by Carneiro and Fava-de-Moraes (1965).

The comparison of autoradiographs from animals killed 4 hours, 5 days and 9 days after labelling provided significant new information for the examination of transalveolar fibres. The dense line of silver grains over specific areas of alveolar bone surface was indicative of active bone formation. These formative areas provided the basis for determining the physiologic distal, buccal and occlusal direction of migration of mouse molars relative to the alveolar bone. Furthermore, bone was deposited around collagen fibres. In this manner, periodontal fibres were incorporated as Sharpey fibres at the depository bone surfaces (Fig. 5). In animals killed 5 or 9 days after labelling, areas showing Sharpey fibres extending uninterrupted into the ligament as periodontal fibres were directly correlated with areas of bone apposition (Figs. 7, 8, 19, 20). Sharpey fibre patterns could therefore be used directly to interpret the direction of tooth drift.

The autoradiographs were essentially two dimensional figures. Furthermore, autoradiographs of serial sections have not been previously reported. Consequently, the studies which report autoradiographic

evidence of tooth drift, do not fully describe the movements. Stallard (1963) describes distal and occlusal migration but not buccal movement of mouse molars. Tonna (1976a, 1976b) reports a similar rate of bone matrix production at the alveolar crest of 35 day old mice as was found in this study. His findings of between 2.13 and 4.56 μm per day are similar to the daily rate of 4-4.5 μm found in this study. However, Tonna reports an examination of the maxillary molars of mice and his topographical maps of bone deposition are therefore not comparable with the results of this study.

Another significant finding from the autoradiographs in this investigation was that cellular cementum apposition was continuous with dentine deposition inside the root canal. Tonna (1976b) schematically illustrates a similar finding. It appeared that cellular cementum deposition was related to the elongation of the root. The periodontal surface of the deposits to which Sharpey fibres attached, seemed to behave like the acellular cementum of the cervical parts of the root.

The technique of autoradiography provided consistent results over similar sections from the same animals. However, quantitative grain counting provides data which must be statistically analysed (Rogers, 1973). The sample size in this examination was inadequate for any statistical comparison of turnover rates based on the different intervals between injection and sacrifice. Autoradiographs of the animal killed 9 days after injection had a higher concentration of silver grains compared with similar areas in the animal killed 5 days after injection (Figs. 19, 20). In these animals all histologic and autoradiographic processing was carried out concurrently. The

difference in grain density may have reflected an unintentional variation in the initial dose of radioactive proline, variation in the net uptake of the injected material by the tissue, or some other physiologic difference between the animals. However, the width of the layer of bone formed in these two animals in similar areas was proportional to the delay between injection and sacrifice. This finding indicated that a comparable rate of bone matrix formation was occurring and therefore the concentration of silver grains reflected a difference in effective dosage to the bone forming cells. Comparison of grain counts from the periodontal ligaments of these animals would be quite misleading.

It is generally recognised that radioactive proline is not a specific label for collagen. Although the exact proportions of radioactivity in each of the tissue components are disputed, all investigators find that some radioactive proline is incorporated into forming collagen (Carneiro and Leblond, 1966; Orlowski, 1976; Rippin, 1976, 1978; Sodek, 1976, 1977; Sodek *et al.*, 1977). In the present investigation silver grains could not therefore be associated solely with synthesis of collagen. Absence of silver grains over specific sites was, however, direct evidence that there was no ongoing synthesis of collagen. In the pilot experiment a heavy concentration of silver grains was observed over the periodontal ligament, pulp, marrow spaces and gingival connective tissues. However, apart from activity over some osteocytes, no silver grains above background levels were observed over the bone matrix in either 23 or 42 day old mice. In particular, there was no autoradiographic evidence of

turnover in the interseptal regions, demonstrated in Fig. 18, which Cohn (1972a) and Dunstan (1975) describe as incorporating transalveolar fibres.

The fact that there was no uptake of label into Sharpey fibres in alveolar bone indicated that there was no remodelling of the intraosseous portions of transalveolar fibres. Moreover, there were no cellular and vascular elements observed in the alveolar bone associated with Sharpey fibres as would have been expected had turnover been occurring. Consequently, further experiments utilizing the greater numbers of animals which would have been necessary to statistically determine turnover rates were not undertaken.

HISTOLOGY

There were several aspects of histologic preparation peculiar to this study. The most significant problem encountered was staining to show Sharpey fibres in normal sections of bone and in autoradiographs.

Pollack's trichrome stain was successful in staining Sharpey fibres red against a green matrix background in routine sections. These results were not reproduced in either pre or post-staining of autoradiographs. Many stains which were tested on autoradiographs had damaging effects on the nuclear emulsion. Haematoxylin and eosin frequently left a fine precipitate of eosin over the section which was difficult to differentiate from silver grains. It was noted that other investigators often use only haematoxylin as a counterstain for autoradiographs (Stallard, 1963; Beertsen and Tonino, 1975).

Van Geison stain seemed to dissolve most silver grains, thus rendering the autoradiographs useless. Acid fuchsin and coomassie blue stained the emulsion giving a crazed appearance to the sections. The most useful histologic stain to combine with autoradiographs in this study was nuclear fast red followed by indigocarmine and picric acid (Mortreuil-Langlois, 1962).

Several light microscope techniques for differentiating Sharpey fibres in unstained sections were also evaluated as it was considered that all stains affect the nuclear emulsion to some degree. Polarized light differentiated Sharpey fibres from the surrounding bone matrix. However, only fibres in one direction were clearly seen for each position of rotation of the stage. Phase contrast produced an image in which the patterns of Sharpey fibres could be seen due to their different orientation from the other bone matrix fibres (Fig. 9). Differential interference contrast gave similar information to phase contrast without a halo effect. The image had a three dimensional character which made the Sharpey fibres appear quite clear (Fig. 10). Differential interference contrast was considered a most valuable addition to the light microscope for studying anatomical features on unstained autoradiographs. The great advantage of the technique would be in quantitative autoradiography when stains are not used and there may be a need for structural identification. Rheinberg's differential colour illumination was spectacular in showing the silver grains over the sections. This could be most useful in quantifying, by photometric analysis, the numbers of silver grains over sections (Fig. 11).

Another feature of the histologic preparation was the need to define the depth and orientation of each section. The three mandibular molar teeth of the mouse did not lie in the one plane. The second molar was inclined more lingually than the first and the third more so than the second. Hence, an axial section through the roots of the first molar did not pass through the long axis of the second and third molar teeth. The second and third molars also had a progressive mesial tilt so that buccolingual sections that were axial at the first molar were oblique at the third molar.

PREDICTION OF SHARPEY FIBRE PATTERNS IN BONE

The exact orientation of sections proved to be very important in this study as it influenced the patterns of Sharpey fibres that were seen. In all the mice, a similar distal, buccal and occlusal direction of drift of the teeth was found. In horizontal section, a depository surface was seen therefore on the mesial and lingual of each root (Fig. 37).

As the tooth moved buccally, the lingual root surface moved away from the bone. At each point on the distolingual and mesiolingual socket surfaces, buccal drift of the tooth relative to the bone translated a narrower section of the root into a given width of periodontal space. Bone deposition on these socket surfaces was therefore necessary to re-establish ligament width. As the teeth moved distally, depository surfaces were similarly formed on the mesial, mesiolingual and mesiobuccal aspects. Conversely, resorptive surfaces were formed distally, buccally and distobuccally

as the tooth advanced, The limit of the depository surfaces on the mesiobuccal and distolingual of each root was determined by the relative amounts of distal and buccal drift (Fig. 37). At positions on the distal alveolar wall where the net increase in periodontal ligament width due to buccal movement of the root exceeded the decrease due to distal drift, the surface was seen to be depository. A mesiodistal section taken from the lingual side of the mandibular molar segment consequently showed a depository surface on both the mesial and distal of the interseptal bone (Fig. 20). Sections taken further to the buccal had a resorptive surface on the mesial of the interseptal bone and a depository surface on the distal (Fig. 8).

Buccolingual sections demonstrated the same feature. Sections taken through the distal of a root (Fig. 38), showed deposition only on the lingual side. The buccal socket wall had a resorptive surface as the root was moving distally and buccally towards it. Conversely, sections through the mesial of a root (Fig. 39) showed bone deposition on the lingual side, associated with buccal drift, and on the mesiobuccal aspect associated with the net increase in periodontal width as the tooth moved distally.

SHARPEY FIBRES OR TRANSALVEOLAR FIBRES?

Developmentally the formation of tooth to tooth fibres, which were subsequently incorporated into alveolar bone, was not observed. This feature was clearly shown in the 23 day old mice. A distinct reversal line was seen in the interdental septum between Sharpey fibre bone of the second and third molars (Figs. 30, 31). A similar

reversal line was not seen between the first and second molars which developed the interdental crest concurrently (Fig. 33). In this area, Sharpey fibres from each tooth were inserted to a similar depth. However, in serial sections, Sharpey fibres from each tooth root were seen not to be continuous although they intermingled as a V-shaped pattern in the bone (Figs. 3, 33). In adult mice, a pattern similar to that described by Dunstan (1975, Fig. 2) was seen with Sharpey fibres crossing the interseptal bone (Fig. 35). The variation in observations at different ages led to the study of the development of patterns of Sharpey fibres in bone.

The patterns of Sharpey fibres in bone, determined by the incorporation of periodontal fibres at depository bone surfaces, were correlated with the direction of eruption and drift to the teeth (Figs. 33, 34, 35). This study therefore supported the conclusion of Stein and Weinmann (1925) who state that the direction and rate of migration of the teeth can be assessed from the distribution and arrangement of Sharpey fibre bone.

The observed patterns needed interpretation to enable deduction of the previous eruptive path. As the teeth moved, bone, which was deposited in specific areas to maintain the width of the periodontal space, progressively incorporated periodontal fibres as Sharpey fibres (Fig. 41). Furthermore, the spatial organisation of the periodontal ligament was observed to be moved with the teeth. Deposition of bone around any fibre which was not in the line of tooth movement resulted in that fibre being associated, at the new bone surface, with a different level of the root and periodontal

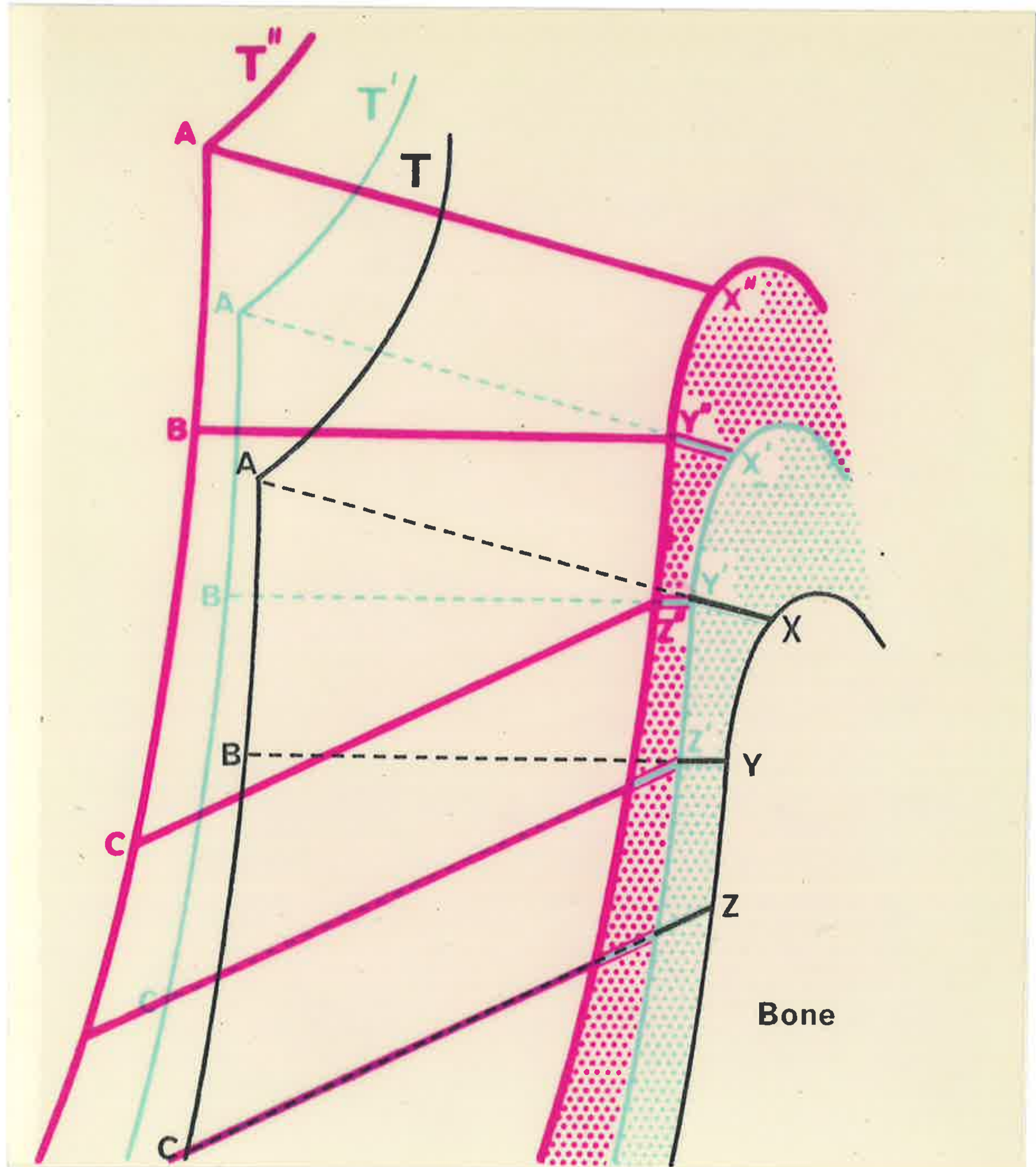

Fig. 41

Schematic diagram showing the formation of Sharpey fibre patterns due to eruption and distal movement of a tooth, T, relative to alveolar bone. The periodontal fibres AX, BY, CZ, attached to the tooth at A, B and C respectively, maintain their relation to the tooth as it migrates to positions T' and T" and are designated on the green and red overlays as AX',BY', CZ' and AX", BY", CZ". The relation of the alveolar bone to the tooth is maintained by surface bone apposition, shaded. As the tooth moves from T to T', green overlay, the bone incorporates parts of the periodontal fibres AX, BY and CZ as Sharpey fibres. At position T', there is remodelling in the periodontal ligament and the oblique fibre, still attached to the tooth at green C, becomes attached to the bone at point Z', forming a new periodontal fibre CZ'. The fibre at Z' previously formed part of the horizontal fibre BY (black) at position T. At position T", red overlay, the oblique fibre, now at red C, is attached to the bone at Z" which was previously part of the horizontal fibre BY' (green) and before that the alveolar crest fibre AX (black). A curved fibre pathway XY'Z" is thus formed in bone. Fibre remodelling in the ligament forms an essential part of this process.

ligament. Further tooth movement, leading to further bone deposition continued the process so that the direction of a Sharpey fibre deep in bone often was quite different from the direction of the periodontal fibre with which it was still continuous. The redirection of an alveolar crest fibre to an oblique fibre has been illustrated schematically in Fig. 41 and anatomically in a 23 day old mouse in Fig. 33.

During the distal, buccal and vertical drift, with the teeth in occlusion, the direction of the alveolar crest fibres from the distal tooth roots was observed to approximately parallel the drift path. These fibres were therefore incorporated as straight fibres. Furthermore, they were noted in some areas to coincidentally parallel the oblique fibres of the adjacent periodontal ligament (Fig. 35).

Weinmann and Sicher (1955), Frank *et al.* (1958), Selvig (1964, 1965) and Shackleford (1971, 1973) all contribute to the understanding that Sharpey fibres in bone are unmineralized or have unmineralized cores. Along the length of a Sharpey fibre, the degree of calcification varies (Boyde and Jones, 1968; Shackleford, 1973; Jones and Boyde, 1974). Consequently these fibres cannot be considered to be mobile within bone.

In this study periodontal fibres were seen to become incorporated into bone matrix (Fig. 5). Furthermore, no remodelling of Sharpey fibres in bone occurred. On autoradiographs, the portion of a periodontal fibre which was soon to be incorporated into bone appeared to be less active than fibres in the centre of the ligament (Fig. 5). Sharpey fibres were neither labile nor mobile in bone. However, they

maintain continuity with periodontal fibres in the ligament attached to the tooth moving away from the depository bone surface. It was concluded, therefore, that a mechanism for fibre elongation and remodelling must have existed in the ligament between the depository bone surface and the tooth. Quigley (1970) and Cohn (1972b) state that the presence of transalveolar fibres is indicative of the existence of an intermediate plexus in some form. However, they fail to relate the patterns of transalveolar fibres to distal drift.

Cohn (1972a) and Dunstan (1975) both report collagen fibre patterns in adult mice. In the adult mice of the present study, as long as the mesiodistal sections were taken in the midroot region or towards the buccal side, similar patterns of Sharpey fibres passing from one side of the interdental septum to the other were seen (Fig. 35). However, this pattern was shown to be formed by the translocation of Sharpey fibres from a depository to a resorptive surface due to occlusal, distal and buccal drift.

Furthermore, no evidence of selective reattachment of periodontal fibres to the exposed ends of Sharpey fibres at resorptive surfaces was found (Fig. 36). Reattachment to resorptive surfaces was observed to be by spot deposition of bone around fibres in contact with the resorbed surface. At resorptive surfaces, at any one time, there were areas of resorption and reattachment. These concurrent processes are described by Lefkowitz and Waugh (1945), Enlow (1968) and Baron (1973a). Kraw and Enlow (1967) suggest that some of the intrinsic fibres of the resorbed margin may become involved with reattachment. However, they emphasize that the Sharpey fibres, which originate

elsewhere, play no role in reattachment. Matena (1973) refers to a chelating effect by osteoclasts which demineralizes collagen fibres and leaves them exposed to join periodontal fibres. In the current investigation, a distinct reversal line was usually seen between Sharpey fibre bone formed at different times (Fig. 31). No conclusion was made on the role of intrinsic bone matrix fibres in reattachment.

On the lingual side of the teeth, due to buccal drift, periodontal fibres were incorporated as Sharpey fibres. These also traversed the entire thickness of the coronal alveolar wall but were seen clearly to terminate at the periosteum (Fig. 40). Furthermore the ease with which periosteum can be elevated on the lingual and buccal of teeth as a smooth homogenous layer indicates that this layer is not bound firmly to bone matrix by collagen fibres. Periosteal bone was observed over the more apical areas of the lingual cortex (Fig. 40).

The processes involved in the formation of Sharpey fibre patterns have been described prior to Cohn's descriptions of transalveolar fibres (Enlow, 1968). Cohn shows that Sharpey fibres are generally uninterrupted in bone except at reversal lines and marrow spaces (Cohn, 1972b, 1975). However, he does not correlate the uninterrupted Sharpey fibres with areas of bone deposition and physiologic drift. His schematic diagram (Fig. 1) shows Sharpey fibres to be continuous with periodontal fibres from adjacent tooth roots. This finding was not seen in serial histologic sections in the present study and was therefore disputed. Furthermore, the photomicrographs in his own

publications (Cohn 1972a, 1972b) do not show tooth to tooth fibres perforating alveolar bone. Rather, fibres from one tooth are seen to enter the bone at a depository surface and terminate at a resorptive surface in the same manner as was shown in the present study (Fig. 35). Bernick *et al.* (1974) are also unable to trace tooth to tooth fibres perforating alveolar bone in marmosets.

Another major problem with Cohn's concept is its failure to recognise the radial nature of periodontal fibres around each tooth (Fig. 37), and to consider the paths of Sharpey fibres not directed towards adjacent teeth or periosteum. The present study found that Sharpey fibre attachments were formed in association with each root on a pre-existing trabecular framework and in the line of the periodontal fibres. These trabeculae formed primary Haversian systems, but little secondary remodelling was observed. The translocation of bone due to physiologic drift seemed to provide adequate remodelling of the coronal parts of the alveolus which were formed as lamellar bone incorporating Sharpey fibres. Baron (1973a) also refutes Cohn's concept although his study is not primarily of transalveolar fibres. Baron (1973a) studies alveolar bone remodelling due to physiologic drift and finds, as in the present investigation, that Sharpey fibre patterns are formed in bone due to tooth drift. He also fails to confirm in serial sections the presence of tooth to tooth fibres perforating alveolar bone.

RELATION OF TRANSEPTAL FIBRES TO TRANSALVEOLAR FIBRES

Edwards (1975) suggests incorporation of transeptal fibres into the forming alveolar bone crest as a possible method of formation of transalveolar fibres.

Cohn (1957), Eccles (1959) and Trott (1962) show that the transeptal ligament forms as the teeth erupt. Ten Cate (1969) states that transeptal fibres form from the frayed ends of the investing layer over the crown as it erupts. At this stage of development the interdental crest is above the cemento-enamel junction. Moreover, following eruption the distance between the interdental crest of bone and the cemento-enamel junction slowly increases with age (Belting *et al.*, 1953; Stallard, 1963; Cohn, 1965, 1966; Goldman and Cohen, 1973). Hence, even though there is continuous eruption and growth of the septal bone, it would be expected on a developmental basis that the transeptal ligament would be carried with the teeth and not become incorporated into alveolar bone. No evidence of incorporation of transeptal fibres into alveolar bone was found in the study.

SHARPEY FIBRE PATTERNS, PERIODONTAL FIBRE ARRANGEMENT AND FUNCTION

Physiologic drift in rat teeth is related to a functional repositioning of the molar segments to maintain optimal position relative to the masticatory muscles as the jaw is translated forward during growth (Sicher and Weinmann, 1944). Interpretation of Sharpey fibre patterns and autoradiographs of the mice of this study revealed that drift was in a distal, buccal and occlusal direction, similar to the rat.

Prior to eruption, the teeth were directed occlusally with a slight lingual and mesial tilt. During eruption, the roots continued to elongate apically and bone was deposited on the alveolar crests. Below the alveolar crests there was remodelling on the socket walls to maintain ligament width. Various patterns of bone deposition were seen as the irregularly tapering roots moved occlusally relative to the bone (Fig. 13).

The rapid eruptive phase developed a particular pattern of Sharpey fibres in bone (Fig. 33). After the teeth were in occlusion, the animals continued to grow. Associated with growth of the jaw, the teeth moved distally, buccally and occlusally, with overall greater movement of the crowns than of the apices. The movements were a continuation of the eruptive path and gradually changed the patterns of Sharpey fibres in bone (Figs. 34, 35).

Miura *et al.* (1970) observe that the most rapid movement occurs in rat molars in the pre-eruptive and pre-functional phases although the teeth continue to move distally at a diminishing rate throughout life. Tonna (1976b) and Stahl and Tonna (1977) find that the rate of alveolar bone formation in mice diminishes with age. The distal, buccal and occlusal physiologic migration of the teeth could therefore be considered as a continuing eruption at a diminished rate throughout life.

A conspicuous difference in the arrangement of the periodontal ligament on the mesial and distal of the teeth is noted in experiments by Miura *et al.* (1970). On the mesial side of the roots, the ligament

appears more organised with fibres and cells aligned as if under a tensional force. Deep Sharpey fibre insertions are seen on this side of the septum. Conversely, the periodontium on the distal of the roots appears less organised with fewer major fibre bundles and no Sharpey fibre insertions.

The difference in the appearance of the tissue organisation on each side of the roots was confirmed in this study. A major factor in the difference was the appearance of long Sharpey fibres, continuous with periodontal fibres, at depository bone surfaces on the mesial and lingual of each tooth. Apparently the ligament maintained its normal functional capabilities in mastication despite obvious variation in its structure on each side of the teeth. The role of other elements of the periodontium in transmission of the masticatory loads is an area requiring further investigation. The possible role of proteoglycans and glycoproteins in the viscoelastic properties of the periodontal ligament is recognised (Melcher and Walker, 1976; Mickelites and Orlowski, 1977).

Dunstan (1975) reports a similar difference in organisation of the periodontium of the mouse on the mesial and distal of the teeth. He suggests that this may reflect adaptation to a net distal force vector generated functionally. This is consistent with the proposal of Rodbard (1970) that orientation of collagen in tissues reflects the tensional demands on the tissue. The direction of drift appeared in this study to be a continuation of the normal eruptive path. The relative contributions of environmental forces and normal eruption

to the distal, buccal and occlusal migration of mouse molars was not clarified by the present investigation.

Sharpey fibre patterns were found to be a static reflection of previous tooth movements relative to the alveolar bone. The patterns seen in bone were considered not be an extension of the functional periodontal fibre system as transalveolar fibres, in the manner described by Cohn (1972a) and Dunstan (1975).

McCutchen (1975) states that collagen is the prime tension carrier in bone. Baumrind (1969) shows that bone will bend under masticatory forces. The role of Sharpey fibres during function is questioned by Boyde (1972) who recongises that the presence of uncalcified collagen fibres within bone may impart special properties. Much of the matrix of the coronal part of alveolar bone was composed of Sharpey fibres. Little remodelling of Sharpey fibre bone in these areas was observed in this study. Sharpey fibres would seem to have a structural role as well as a role in attachment. However, the relation of their structural organisation to the function of the bone was not ascertained.

The arrangement of intrinsic fibres within bone is ordered by unknown processes; piezo electricity (Bassett, 1965), osteocytic processes (Boyde and Jones, 1968), tension or other. However, they are considered to be functionally orientated and it was considered significant that, whilst being incorporated into bone, at the periodontal interface Sharpey fibres were parallel to the periodontal fibres.

It may be that Sharpey fibre orientation was suited to aid distribution of functional loading of teeth to the alveolar bone. The evidence from this study indicated, however, that the process of load distribution was most likely to be associated only with those fibres at the bone periodontium interface. The orientation of deeper parts of fibres was often quite different to the orientation of more superficial parts or to the periodontal fibres (Fig. 33). It was also observed that many areas of the periodontium on resorptive surfaces were attached by only shallow areas of spot deposition (Fig. 36).

Sharpey fibre bone is formed in areas of tension during orthodontic tooth movements (Reitan, 1959; Edwards, 1968; Azuma, 1970). Salzmann (1965) states that Sharpey fibre bone formed due to orthodontic tooth movement must be remodelled before teeth will be stable following treatment. However, in this study a process of remodelling of Sharpey fibre bone did not appear to be active. Any remodelling of Sharpey fibre bone involved replacement with secondary Haversian systems. Sharpey fibre patterns were not remodelled in bone.

The influence of Sharpey fibre orientation on the stability of tooth positions or on the functional resistance of the alveolar process to load is an area requiring further research. However, the failure to identify a physiologic system to remodel the Sharpey fibre patterns suggests that their role is structural once incorporated into bone and not related to the dynamic and rapidly remodelling periodontal ligament.

CHAPTER 7

CONCLUSIONS

1. Remodelling of Sharpey fibres in bone was not demonstrated in this investigation. Once incorporated into bone, Sharpey fibres formed part of the bone matrix and were resorbed coincidentally with the intrinsic bone matrix fibres at either endosteal, periosteal or periodontal surfaces.

2. Autoradiography using tritiated proline proved a sensitive vital bone marker which showed the distal, buccal and occlusal direction of migration of mouse molars. The movement of the crowns was greater than that of the apices which maintained a close relationship to the inferior alveolar nerve.

3. Sharpey fibres were formed by deposition of bone around principal periodontal fibres. At depository or resting surfaces Sharpey fibres maintained continuity with their associated periodontal fibres. At resorptive surfaces, attachment of periodontal fibres was only to the surface of bone.

4. Areas where Sharpey fibres were continuous with periodontal fibres correlated with the depository bone surfaces depicted by vital bone marking. The patterns of Sharpey fibres in bone therefore reflected the direction of drift of the teeth. Patterns of Sharpey fibres in bone were not correlated with the spatial orientation of periodontal fibres.

5. In specific areas where the alveolar partitions were thin, due to tooth drift, Sharpey fibres which were incorporated at depository surfaces were progressively translocated to resorptive periodontal or periosteal surfaces. Sharpey fibres in this situation consequently traversed the crestal third of interdental or lingual bone. They were not observed to extend into either the periodontal ligament or periosteum. This was a direct contradiction of the features of transalveolar fibres proposed by Cohn (1970).

6. The name transalveolar fibres, was concluded to be a misnomer applied to normal patterns of Sharpey fibres in alveolar bone of adult mice. Developmentally, each tooth formed its own Sharpey fibre attachments independently. Tooth to tooth fibres, formed in the supracrestal tissue, were not incorporated into the growing bone crests. Selective reattachment of periodontal fibres to the exposed ends of Sharpey fibres at resorptive surfaces did not occur.

7. As periodontal fibres became trapped in the bone as Sharpey fibres, and teeth migrated away from the bone in a distal, buccal and occlusal direction, fibres must have been remodelled in the periodontal ligament to maintain the observed functional orientation of principal periodontal fibres.

8. A primary function of the gomphosis was to allow for relative movements of the teeth, both with respect to each other and to the investing alveolus.

9. Sharpey fibres formed a significant proportion of alveolar bone matrix as well as playing a role in attachment of teeth to bone.

10. The influence of Sharpey fibres in the matrix of bone, in the physiologic behaviour of bone, was suggested as an area requiring further research.

CHAPTER 8

APPENDICES

I. SOLUTIONS FOR INJECTION

Concentration of Stock Solutions

L-proline .... 2.5×10^{-5} mol/L

1-[3,4(n)-^{3}H] proline .... 2.5×10^{-5} mol/L

L-[3,4(n)-^{3}H] proline .... 1.0 Ci/L

Specific Activity of L-[3,4(n)-^{3}H] proline ..... 4×10^4 Ci/mol

Solution A for animals between 10 and 15 grams

15% dilution of L-[3,4(n)-^{3}H] proline stock solution.

Concentration of solution A

$$= 15 \times 10^{-2} \times 1 \text{ Ci/L}$$

$$= 150 \ \mu\text{Ci/ml}$$

Solution B for animals between 20 and 30 grams

30% dilution of L-[3,4(n)-^{3}H] proline stock solution.

Concentration of solution B

$$= 30 \times 10^{-2} \times 1 \text{ Ci/L}$$

$$= 300 \ \mu\text{Ci/ml}$$

Solution C, control solution for animals between 20 and 30 grams

30% dilution of L-proline stock solution.

Concentration of solution C

$$= 30 \times 10^{-2} \times 2.5 \times 10^{-5} \text{ mol/L}$$

$$= 7.5 \times 10^{-6} \text{ mol/L}$$

II. 23 DAY OLD MOUSE EXPERIMENT

MOUSE CODE	AGE	WEIGHT	SOLUTION INJECTED AND DOSE	VOLUME INJECTED	INTERVAL BETWEEN INJECTION AND SACRIFICE	DECALCIFICATION	
M1 EXPT	23 days	13 gm	Solution A (Appendix I) 5 μCi/gm body wt. = 65 μCi	Concentration Solution A = 150 μCi/ml Vol $\frac{65}{150}$ ml = .433 ml	4 hours	R	E.D.T.A.
						L	Formic Formate
M2 EXPT	23 days	13 gm	Solution A (Appendix I) 5 μCi/gm body wt. = 65 μCi	Concentration Solution A = 150 μCi/ml Vol $\frac{65}{150}$ ml = .433 ml	4 hours	R	E.D.T.A.
						L	Formic Formate
M3 CONT	23 days	11 gm	Isotonic Sterile saline	Vol = .366 ml	4 hours	R	E.D.T.A.
						L	Formic Formate
M4 CONT	23 days	11.5 gm	not injected			R	E.D.T.A.
						L	Formic Formate

III. 42 DAY OLD MOUSE EXPERIMENT

MOUSE CODE	AGE	WEIGHT	SOLUTION INJECTED AND DOSE	VOLUME INJECTED	INTERVAL BETWEEN INJECTION AND SACRIFICE	DECALCIFICATION
M5 EXPT	42 days	25.5 gm	Solution B (Appendix I) 5 μCi/gm body wt. = 127.5 μCi	Concentration Solution B = 300 μCi/ml Vol $\frac{127.5}{300}$ ml = .425 ml	4 hours	R E.D.T.A. L E.D.T.A.
M6 EXPT	42 days	24.8 gm	Solution B (Appendix I) 5 μCi/gm body wt. = 124 μCi	Concentration Solution B = 300 μCi/ml Vol $\frac{124}{300}$ ml = .413 ml	4 hours	R E.D.T.A. L. E.D.T.A.
M7 CONT	42 days	25.6 gm	Solution C (Appendix I) 1.25×10^{-10} mol/gm body wt. $= 3.2 \times 10^{-9}$ mol	Concentration Solution C $= 7.5 \times 10^{-6}$ mol/L Vol $\frac{3.2}{7.5} \times 10^{-3}$ L = .427 ml	4 hours	R E.D.T.A. L E.D.T.A.
M8 CONT	42 days	25.5 gm	Solution C (Appendix I) 1.25×10^{-10} mol/gm body wt. $= 3.18 \times 10^{-9}$ mol	Concentration Solution C $= 7.5 \times 10^{-6}$ mol/L Vol $\frac{3.18}{7.5} \times 10^{-3}$ L = .424 ml	4 hours	R E.D.T.A. L E.D.T.A.

IV. 42 DAY OLD MOUSE EXPERIMENT

MOUSE CODE	AGE	WEIGHT	SOLUTION INJECTED AND DOSE	VOLUME INJECTED	INTERVAL BETWEEN INJECTION AND SACRIFICE	DECALCIFICATION
M21 EXPT	42 days	24.8 gm	Solution B (Appendix I) 5 μCi/gm body wt. = 124.0 Ci	Concentration Solution B = 300 μCi/ml Vol $\frac{124}{300}$ ml = .413 ml	5 days	R FORMIC FORMATE L E.D.T.A.
M22 EXPT	42 days	25.5 gm	Solution B (Appendix I) 5 μCi/gm body wt. = 127.5 μCi	Concentration Solution B = 300 μCi/ml Vol $\frac{127.5}{300}$ ml = .425 ml	9 days	R FORMIC FORMATE L E.D.T.A.
M23 CONT	42 days	21.5 gm	Solution C (Appendix I) 1.25×10^{-10} mol/gm body wt. = 2.69×10^{-9} mol	Concentration Solution C = 7.5×10^{-6} mol/L Vol $\frac{2.69}{7.5} \times 10^{-3}$ L = .359 ml	5 days	R FORMIC FORMATE L E.D.T.A.
M24 CONT	42 days	27.2 gm	Solution C (Appendix I) 1.25×10^{-10} mol/gm body wt. = 3.40×10^{-9} mol	Concentration Solution C = 7.5×10^{-6} mol/L Vol $\frac{3.4}{7.5} \times 10^{-3}$ L = .453 ml	9 days	R FORMIC FORMATE L E.D.T.A.

CHAPTER 9

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