OSTEOGENESIS IN THE HEALING OF THE EXTRACTION WOUND

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PRECIS

In a preliminary study, it was found that certain features in the healing of the extraction wound in experimental animals were inconsistent with conventional descriptions. In addition to the formation of new bone within the alveolar socket, bone was also formed subperiosteally on the buccal surface. The present investigation is an extension of the above study.

A hooded strain of the Norwegian rat in which a maxillary molar was removed has been used, and the healing process that followed was studied with the use of:-

1. Intra-vital staining of new bone with
   (a) demethyl-chlortetracycline,
   (b) chlorazol fast pink.
2. Localization of mitotic activities with colchicine.
3. Some histo-chemical observations of extra-cellular components which were also made along with routine histological techniques.

The results indicate that there is a close parallel between healing in fractures, in cortical defects in bone and in the alveolar extraction wound, by the formation of "periosteal", "endosteal" and "uniting" callus.

However, the formation of buccal periosteal bone in the extraction wound was a variable feature and appeared to be related to the presence of inflammation. Inflammation in the periosteum could arise from (a) a direct extension from the oral wound, or (b) indirectly across the alveolar socket wall. The lack of periosteal bone formation in the nasal and palatal surfaces of the maxilla cannot be explained.

Comparison with repair of bone in other situations suggests that under certain circumstances, inflammation will lead to periosteal bone formation.

Food impaction and sequestration of necrotic bone and/or root fragments leads to intensive inflammation in the socket. This intensive inflammation delayed wound healing and bone formation in the socket.

Endosteal bone formation, analogous to the endosteal callus in the healing of a fracture, was also noted in some specimens.

DECLARATION

This thesis is submitted in fulfilment of the requirements for the Degree of Master of Dental Surgery in the University of Adelaide. Candidature for the Degree was satisfied by obtaining the Honours Degree (First Class) of Bachelor of Dental Surgery in 1965.

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

The results of the present investigation have been presented in part to the meeting of the International Association for Dental Research (Australian Section) 1967.

CHAU Kai-Kin

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INTRODUCTION

Although wound healing is the basis of surgical science the intricate mechanisms of this essential process are by no means understood. It is not until recently that attempts have been made to correlate the histological, biochemical and physical aspects of wound healing.

Because of its hard physical nature, bone tissue poses special problems which have, for a long time, defied the application of experimental methods which have yielded results in investigations in healing of soft tissues.

Under normal conditions, wounds of the mouth heal rapidly and without measurable systemic effects. Relatively few detailed investigations, either clinical or experimental, have been concerned with this subject.

Discrepancies in the literature regarding the histogenesis of various tissues associated with alveolar socket wound healing prompted a preliminary investigation by the author. As an outcome of this, certain features in the healing of the extraction wound were noted which called for further investigation.

The present investigation is an attempt at applying some techniques of studying calcified tissues to the healing of

extraction wounds. At the same time, the wound has also been studied from the histological and histochemical angle. An attempt was made to compare and correlate the healing of this intra-oral wound with the healing of a fracture in long bones and healing of cortical defects in bone.

The effect of feeding madder root on the bones of pigs, first observed during the middle of the eighteenth century, gave anatomists of that period a tool for following the development of and repair processes in bone. The basic observations of early workers are still valid, and constitute the foundation of cellular bone physiology as it exists today. The techniques of intra-vital staining of bone using various markers are refinements of the same principle and these form the first part of the present investigation.

In recent years many methods of tagging and labelling cells have become available with the use of radio-active isotopes and have afforded a means of studying the origin and fate of cellular components in tissues. Less accurate and sophisticated methods have also been available which enable a limited insight into cellular activities. One of these latter methods, the use of colchicine, has been applied in the present investigation to observe the mitotic activities of various cellular components in bone repair.

The role of the ground substances and the extracellular components have been emphasised in recent studies on wound healing. Histochemical methods afford a means of identifying various biochemical components present in the healing wound and form a subsidiary approach to the present study of osseous repair.

In the first section of this thesis, the relevant literature on various aspects of bone repair and osteogenesis are reviewed. The theoretical basis of methods used in the present investigation is discussed. The second section reports the methods and findings.

CHAPTER ONE

OSTEOGENESIS IN BONE REPAIR

Urist and Johnson (1943) stated that between Hippocrates' time and 1940 more than 4000 works were published on the problem of the healing of fractures. With the recent increase of interest in skeletal physiology, there has been considerable further addition to the literature concerning the reaction of bone tissue to trauma. Important advances in the basic mechanisms of bone formation and calcification have been made. These fundamental issues have been dealt with in detail in several monographs (Neuman and Neuman, 1958; Sognnaes, 1960; McLean and Urist, 1961; Blackwood, 1964; Richelle and Dallemagne, 1965) and are beyond the scope of the present review. An attempt is made in this review to draw attention to the principal features of previous development in the field of osseous repair as far as is considered pertinent to the subject of this thesis.

In the repair of soft tissue wounds, necrotic tissue and blood clot are removed by macrophages and giant cells and replaced by vascular connective tissue formed by invading fibroblasts and blood vessels. The new tissue

matures into a fibrous 'scar' which is subsequently remodelled to harmonize anatomically and functionally with the normal connective tissue of the region. The significance of the histological, biochemical and physical sequence of events in wound healing are now beginning to be understood and attempts at correlating these various aspects made (Edwards and Dunphy, 1958; Perez-Tamayo, 1961; Florey and Jennings, 1962; Chen and Postelthwait, 1964).

The repair of a wound involving bone proceeds along essentially similar lines to that of soft tissue except that the damaged region is organized by osteogenic cells as well as fibroblasts, and the resulting scar tissue, or 'callus' contains new bone as well as fibrous tissue and often cartilage as well.

In the early stages of osseous repair, it is obvious that the new bone laid down is derived from osteoblasts. In the later stages, however, there is good evidence that the fibrous tissue in the vicinity of the osseous damage may be induced to form fibro-cartilage and bone. The general circumstances in which the 'induction' of fibrous tissue reveals its osteogenetic pctential are not well understood (Bridges and Pritchard, 1958; Bridges, 1959; Moss, 1960).

I. HISTORICAL AND CURRENT CONCEPTS OF BONE REPAIR

Ever since antiquity there has been controversy over which tissue element plays the chief part in fracture healing. Hippocrates maintained that only the bone marrow was callus-forming. Galen considered that the fracture was joined by some undefined cement-like substance which, however, was never transformed into true bone.

By and large these views were unopposed until the 18th Century, when Duhamel (1742-1743) showed, by means of madder feeding, that new osseous tissue was deposited under the periosteum and so argued that the periosteum is responsible for osteogenesis. John Hunter (1770) strongly opposed this view and claimed that new bone came from the arteries in the bone and its surroundings, and that the periosteum has no osteogenic capacity.

During the two centuries that followed, this field was dominated by arguments for and against the "periosteum theory". Were specialised cells, either those from the periosteum or those lining the bone trabeculae, the only antecedents of the new bone or were any mesenchymal cells, when properly stimulated, transformed into osteogenic tissue?

A. The Role of the Periosteum and Bone Cells

In 1864, Gegenbaur gave the first description of osteogenic cells; he called them osteoblasts and thought that they were specific cells lying in the cambium layer of the periosteum, the marrow cavities and lamellar systems.

Ollier (1867) by transplanting viable autogenous bone into intra-muscular and intra-osseous sites, demonstrated the transformation of the periosteal cells into osteoblasts which then laid down new bone on the surface of the implant.

On the other hand, Macewen (1912) did not consider the cells of the periosteum as a source of new bone. He called the periosteum a "limiting membrane" and assigned to it the major function of being a source of vascular supply to bone. He regarded the viable bone to be the chief source and origin of the callus associated with fracture healing.

G. Axhausen (1908, 1909) from studies of autogenous bone grafts, reported that new bone formation was not only due to the proliferation of cells of the periosteum, but that cells of the endosteum and the bone marrow also contributed to new bone formation. The osteogenetic capacity of the skeletal tissues when

transplanted into various sites has since been repeatedly demonstrated (Mayer and Wehner, 1914; Mayer, 1919; Nathan, 1921; Kearns, 1934; McGaw and Harbin, 1934; Bertelsen, 1944; Ham and Harris, 1956; Ray and Holloway, 1957). Different authors attach varying degrees of importance to the different tissue elements. Wehner (1920), Kolodny (1923) and Cowan (1928) emphasised the importance of the layer of osteoblasts in the periosteum, whereas McGaw and Harbin (1934), for example, considered that the endosteal cells and undifferentiated bone marrow cells have at least a function as important as that of the periosteum.

An overwhelming majority of the investigations carried out during the last few decades nevertheless indicate that the periosteum is of primary importance for regeneration in fracture healing.

Great cellular activity in the cambium layer of the periosteum is observed as early as 24 hours after fracture and this continues as long as repair of the fracture is proceeding (Ham and Harris, 1956).

From experimental results in rats it is known that if the periosteum is removed with the bone, fibrous union persists

where the fracture gap is more than 2.0 mm. in width (Pritchard, 1946; Mulholland, 1959). However, in the presence of an intact periosteum covering the fracture gap, rapid bony union results in fractures where up to 17 mm. of rib was removed (Mullholland, 1959; McClements, Templeton and Pritchard, 1961).

Thus, in the early stages of repair, it is clear that the collars of callus which form around the two fragments have their origin from the osteogenic layer of the periosteum. But, as the collars continue to grow and approach one another, there is histological evidence for other possible origins of callus tissue.

The fibrous layer of the periosteum fades out toward the summit of each collar, and the gap between the two collars, which is not separated from muscular tissue by a distinct fibrous layer of periosteum, is invaded by a large number of cells the origin of which is obscure.

One view is that they are osteogenic cells which have emigrated from the collars; another is that they are fibroblasts from the connective tissue elements of the surrounding region (Ham and Harris, 1956).

Since proper callus tissue later appears in this site, it must be questioned whether or not fibroblasts can participate in osteogenesis. There are many different views on this matter. Some authors see no fundamental distinction between the inherent abilities of fibroblasts and osteoblasts to form bone, and stress the pluri-potential of mesenchymal tissues in response to functional requirements. Thus, they believe that the environment rather than the cell is the important factor in determining bone formation. Others consider that osteogenic cells have an inherent tendency to form bone but that fibroblasts also can be induced to do so by certain as yet not clearly understood factors.

B. Connective Tissue Metaplasia and the Theory of Induction

The idea that mesenchymal cells other than osteoblasts may form bone is not new. As early as 1901, Marchand postulated that osteoblasts were formed from mesenchymal cells. Heterotopic bone formation was known to occur in a variety of clinical and experimental situations, for example, the formation of bone in sclerotic aortas, and in repair in the kidney of dogs following partial removal. Huggins (1931) transplanted bladder, ureter and renal pelvis subcutaneously, to muscle, and to the fascia lata, and in each case,

demonstrated the formation of spongy bone with a hematopoietic marrow. He concluded that the proliferating mucosa of the kidney, ureter and bladder in some way stimulated the heterotopic formation of bone.

In the field of osseous repair, Baschikirzew and Petrow (1912) introduced the concept of fibrous tissue metaplasia to explain how new bone was formed in bone transplantation experiments. Leriche and Policard (1928) further elaborated this hypothesis. According to them, undifferentiated "connective tissue cells" in the region of the callus are converted in some unknown way into an osteoid tissue which is then calcified.

This concept received further support from the experiments of Orell who reported bone formation occurring around transplants which had been prepared by boiling, after 100 days (Orell, 1934), and by freezing (Engstrom and Orell, 1943) after 53 days. This led to the "inductive hypothesis" whereby the recipient bed, stimulated by an inductor substance in the transplant, is able to elaborate bone.

Urist and McLean (1952) noted that their anterior eye chamber implants of cortical bone, devoid of viable cells, produced a small deposit of new bone after a latent period

of four weeks. The new bone did not appear to arise from any transplanted osteoblasts, but from ingrowing perivascular connective tissue cells of the host, which established contact with the implant in the process of resorbing it. This has been substantiated by Bridges and Pritchard (1958) who suggested that an inductor factor is released during the resorption of the implant. The results of Ray and Sabet (1963), using tritiated thymidine, are also suggestive of induction in connective tissue by subcutaneous bone grafts.

The precise cellular precursors of these osteogenetic cells from connective tissue is not known. Haggqvist (1929), Wilton (1937) and Trueta (1961, 1963) believed that they originate from vascular endothelium. Gardner (1956), McLean and Urist (1961) believed that spindle cells of marrow and of perivascular spaces may give rise to osteoblasts. Recent work using tritiated thymidine (Tonna and Cronkite, 1961; Young, 1962) demonstrated the evolution of bone-forming cells from undifferentiated, spindle-shaped, mesenchymal or reticular cells.

The stimulus for osteogenic induction is not known. Since the formation of new bone at sites of healing is accompanied by resorption, Leriche and Policard (1928) suggested that the local over-supply of calcium salts

produced as a result of this resorption acts to stimulate osseous metaplasia in the surrounding connective tissue. This has, however, been disproved since the calcium released in areas of bone repair is not utilised locally, but is transported to the systemic circulation. An increased concentration of calcium in the area of healing has not been observed (Cohen et al. 1957; Urist et al. 1958).

Neuhoff (1923) and Rhode (1925) have suggested that some stimulus for example, trauma or infection, was responsible for the metaplasia of connective tissue.

Examination of various types of extracts of bone, bone marrow or periosteum (Levander, 1938; Annersten, 1940; Bertelsen, 1944; Lacroix, 1949; Willestaedt, Levander and Hult, 1950) indicated the existence of certain substances which possess specific osteogenetic qualities --- so called osteogenins. Other authors (Hellstadius, 1947; Heinen, Dobbs and Mason, 1949; Lindahl and Orell, 1951) attributed to these extracts only a non-specific effect.

To determine whether an inductor is liberated from an area of bone repair, Hurley et al. (1959) interposed a millipore filter between the area of healing and the overlying soft tissues. They observed no evidence of bone formation on the soft tissue side of the filter.

On the other hand, Goldhaber (1961) implanted mouse calvaria homografts enclosed in millipore filter diffusion chambers subcutaneously. Using an immunization procedure to discount any effect of cells escaping from the chamber, he noted bone formation on the host side of the chamber. From these studies, Goldhaber concluded that the formation of new bone on the host side of the filter is the result of a "diffusable osteogenic inductor" coming from the new bone laid down on the inner aspect of the filter.

Gelfoam soaked in an aqueous extract of a commercial calf bone paste produced cartilage, osteoid-like tissue and at times bone, when implanted into the anterior chamber of the eye or intra-cranially into rats (Moss, 1958; Anderson et al. 1960). Moss (1960) also reported that freeze-dried preparations of the extracts and a mucopolysaccharide-protein fraction of the extracts were equally effective in stimulating osteogenesis.

Chondroitin sulphate has been shown to accelerate the rate of repair around implants inserted into surgically prepared defects in the skull of rats (Burger, Sherman and Sobel, 1962).

The situation regarding osteogenesis in repair of bone

may be summarised by W. Axhausen's (1956) bi-phasic theory of osteogenesis in which he described bone regeneration in two osteogenetic phases. The first phase originates in pre-existing specific cells and begins after several days. The second phase originates in the non-specific connective tissue and requires several weeks.

W. Axhausen based the evidence for the first phase of his theory on the survival of cells in implants i.e., stimulation of the periosteum and endosteum. The second phase (metaplastic phase) takes place in the surrounding connective tissue. The activating influence in both phases is the necrosis of bone; this has also been suggested by Urist et al. (1958, 1959) and receives some support from Goldhaber's work with diffusion chambers.

In line with W. Axhausen's bi-phasic theory of osteogenesis, Pritchard (1964) introduced the terms 'osteogenic' and 'fibroblastic' blastemas. He defined a 'blastema' as a mass of proliferating, migrating, differentiating and matrix-producing cells. Thus, in the medullary cavity and beneath the cambium layer of the periosteum, an 'osteogenic blastema' develops; while from the fibrous periosteum and extra-periosteal tissues a 'fibroblastic blastema' is formed. The osteogenic blastema possesses immediate bone and cartilage

forming powers by virtue of its already highly differentiated state. The fibroblastic blastema normally confines its activities to collagen fibre production, but seems to have latent osteogenetic powers by virtue of the pluripotentiality of mesenchymal tissues, which may be elicited in special circumstances.

II. MATRIX SYNTHESIS AND MINERALIZATION

Dunphy and his associates (Dunphy and Udapa, 1955; Dunphy, Udupa and Edwards, 1956; Edwards and Dunphy, 1958) by correlating histological, histochemical, biochemical and tensile strength studies on the healing of an incised wound in soft tissues, proposed that there are two phases in wound healing.

The first is a productive or substrate phase which begins shortly after trauma and lasts for about 5 days. During this period, mucopolysaccharides and soluble protein precursors of collagen are accummulated. The second is a collagen phase in which collagen fibres are formed. They regarded the formation of collagen as being the primary factor in re-establishing continuity in the injured tissue. This begins about the fifth day and lasts until completion of healing, during which time, the tensile strength of the wound progressively increases.

In osseous repair, the collagen phase is followed by an osteogenic phase (Udupa and Prasad, 1963) in the third and fourth week, during which time, the granulation tissue is replaced by bone, thus adding strength to the healing bone.

In the formation of collagen, many reports have directly and indirectly indicated the important function of the ground substance (Porter and Vanamee, 1949; Highberger et al., 1951; Dunphy and Udupa, 1955; Taylor and Saunders, 1957). These reports led Fitton-Jackson (1957) to postulate that the fibroblasts secrete a soluble precursor of collagen which is essential for fibre formation, but that the process is not complete without the action of mucopolysaccharides in the ground substance.

Mucopolysaccharides in the ground substance are known to be derived from mast-cells (Asboe-Hanson, 1950) and from the blood stream (Perez-Tamayo and Inhen, 1953). It now seems that fibroblasts themselves are capable of contributing to the total mucopolysaccharide content in the ground substance (Gersh and Catchpole, 1949; Fitton-Jackson, 1954; Grossfeld, Meyer and Godman, 1955; Gaines, 1960).

Collagen and mucopolysaccharides account for the major portion of the organic material in bone, and there is general

agreement that they are elaborated by osteoblasts (Pritchard, 1956; Gersh, 1960; Fitton-Jackson, 1960).

A. Osteoblasts and Ground Substance

The mechanism by which cells synthesize mucopolysaccharides is not understood. There is evidence that many cells of mesenchymal origin secrete these substances during the manufacture of various connective tissue matrices, for example, by fibroblasts (Moore and Schoenberg, 1960), by osteoblasts during fracture repair (Duthie and Barker, 1955b) and by chondroblasts (Godman and Porter, 1960).

Schoenberg and Moore (1958) suggested that osteoblasts synthesize a simple, non-metachromatic polysaccharide into the intercellular space, where it becomes sulphated. In tissue culture experiments, intra-cytoplasmic periodic acid-Schiff (PAS) positive granules during the early phases of bone matrix formation have been noted (Johnson, L.C., 1960; Bassett, 1962). These granules were present just prior to the appearance of extra-cellular metachromasia. This sequence suggests that they are associated in some manner with mucopolysaccharide production.

While these data indicate that acid mucopolysaccharides are elaborated by cells, little is known about the function of these substances in bone formation. Different studies link

them with fibrilogenesis (Jackson and Randall, 1956; Shatton and Schubert, 1954; Meyer, K. 1960), initiation of calcification (Sobel and Burger, 1954), and inhibition of mineralization (Glimcher, 1960).

B. Osteoblasts and Collagen Formation

The hypertrophic Golgi apparatus, the numerous mitochondria and the nucleus displacement as well as the polarity of the osteoblast with respect to the site of matrix formation are compatible with the characteristics of actively secreting cells (Pritchard, 1956). The combination of high alkaline phosphatase activity and cytoplasmic ribo-nucleic acid content shown by the osteoblasts also strongly suggests that a protein is being synthesized and presumably secreted (Pritchard, 1956).

The function of osteoblasts in synthesizing collagen has been clarified considerably by the use of radioactive labelled precursors. Smith and Jackson (1957) reported that osteoblasts in tissue cultures converted C^{14}-L-proline to C^{14} hydroxyproline, a characteristic component of collagen. Leblond et al. (1959), Corniero and Leblond (1959) and Young (1962) observed tritiated glycine first in osteoblasts, then in the surrounding matrix. They concluded that the protein material was synthesised in the cells and subsequently was secreted to form the fibrils.

C. Calcification

The organic matrix is present before evidence of calcification can be detected (Scott and Pease, 1956; Molnar, 1959; Johnson, L.C., 1960). This uncalcified material has been referred to as osteoid.

Some investigators thought that certain changes in this osteoid is required before it can become mineralised. Thomas (1961) proposed that this change lays mainly in the ground substance, which was less highly polymerised in the osteoid. It has been hypothesized that mineralization of an osseous matrix is initiated by some interaction between calcium, phosphate and an as yet unidentified nucleation site or template on the collagen fibril (Glimcher, 1960; Robinson and Sheldon, 1960).

Molnar, (1959), Shatton and Schubert (1954) had shown that a zone of unmineralized collagen and amorphous ground substance surrounds the osteoblast. This zone is in direct contact with a second zone where bone salts are deposited in an amorphous form. In the next layer, the smallest crystals appear to bear a definite relation to an alignment with the 640 Å repeating pattern of the underlying collagen fibrils. This very early stage of ossification can be detected by electron microscopy, but not by standard histological techniques.

CHAPTER TWO

THE HISTOLOGY OF BONE REPAIR

I. HEALING IN FRACTURES OF BONE

Healing in bone has been studied mainly in the healing of fractures. The present day accepted view of the histology in fracture healing is principally based upon the comprehensive studies of Urist and his associates (1941-1943), Ham and Harris (1956), Pritchard and Ruzicka (1950). Allowing for minor differences, these authors are largely in agreement.

A. The Histological Sequence in Fracture Repair.

Weinman and Sicher (1955) divided fracture repair into six histological stages. These, not being distinct entities, show considerable over-lapping within the same fracture area. These stages are:-

(i) formation of a haematoma,

(ii) organization of the haematoma,

(iii) formation of a fibro-callus,

(iv) formation of a primary bony-callus

(v) formation of a secondary bony-callus,

(vi) functional reconstruction of the fractured bone.

A simple account of a fracture repair is as follows (Robbins, 1961):-

"Immediately after a fracture, there is considerable haemorrhage into the fracture site from ruptured vessels within the bone as well as from the torn periosteum and surrounding soft tissues. A haematoma is thus formed that fills the fracture gap and surrounds the area of bone injury. During the 24 to 48 hours that follow, inflammation results in oedema, vascular congestion and an infiltration of leukocytes, chiefly neutrophils.

After two days, the neutrophils are accompanied by a large number of macrophages that begin the phagocytosis of necrotic tissue and red cell debris. At the same time fibroblastic repair is begun which invades the blood clot and forms callus in and about the fracture site.

After the first few days, newly formed bone and cartilage is evident in the fibro-vascular response. In the course of the succeeding days, these bone spicules become sufficiently numerous and aggregated to create a temporary bony union of the fracture. By this time, the inflammatory reaction has largely subsided and the repair is well under way.

In an uncomplicated fracture, the bony callus usually attains its maximal size at about the end of the second or third week. This callus is increasingly strengthened by the precipitation of bone salts, and the widening of the newly formed delicate bone spicules, and is at the same time remodelled by osteoclastic and osteoblastic activity. In such reconstruction, the internal callus which fills the marrow space is also resorbed. If the fracture has been well aligned, virtually perfect reconstruction of the bone is accomplished".

The changes in the periosteum, bone marrow, bone and extra-periosteal soft tissue will now be discussed in turn.

(a) Periosteal Reactions.

Shortly after a fracture the osteoblasts beneath the undamaged periosteum begin to multiply rapidly over a wide area of the surface of the broken bone. These cells are arranged into cords of osteoblasts which deposit trabeculae of woven bone.

Near the fracture, on the other hand, where the periosteum has been damaged, this membrane first becomes congested, oedematous and infiltrated with polymorphonuclear leukocytes. These cells then disappear and there is rapid multiplication

of surviving fibroblasts. Later, the cells are lifted from the bone by a layer of proliferating osteogenetic cells which form beneath them.

Some of these cells differentiate into osteoblasts and osteoclasts which begin the formation of new bone and the destruction of the old bone, but in many of the fractures, some of them differentiate into chondroblasts.

(b) Medullary Reactions.

After a fracture there is always a certain amount of necrotic haemorrhagic marrow in the marrow cavity adjoining the fracture. The adjacent bone is largely necrotic as evidenced by the predominance of empty lacunae. Beyond the necrotic zone, the marrow is congested. Polymorphs, macrophages and osteoclasts assemble at the boundary between the living and dead marrow where they proceed to resorb the necrotic tissue.

Behind the macrophages, there is proliferation of the endosteum and differentiation of cells into osteoblasts. Osteoclasts appear and begin to resorb the wall of the marrow cavity. The marrow osteogenetic cells do not, as a rule, form cartilage in the marrow cavity.

Some days after a fracture, the marrow cavity shows first a zone where the marrow has been displaced by a network of bony trabeculae and then a zone where necrotic marrow is being phagocytosed by macrophages and where the walls of the cavity are being resorbed by osteoclasts.

Similar osteoclastic and osteoblastic reactions are also seen in the vascular spaces of the cortical bone.

(c) Extra-periosteal Reactions.

The soft tissue outside the periosteum shows considerable activity after a fracture. First, there is vascular engorgement and gross oedema with emigration of polymorphs; then there is mobilization of macrophages at the junction of living and necrotic tissues.

This is followed by proliferation of fibroblasts in the loose connective tissue, including that between muscle fibres, in tendon sheaths, and the adventitia of blood vessels. These fibroblasts accumulate around the fracture site, merging with the proliferating fibroblasts of the damaged periosteum.

Between them they are responsible for the formation of a wall of new fibrous tissue around the fracture.

(d) The Fracture Gap.

So far little has been said about the fate of the blood clot and tissue debris in the fracture gap. Debridement is carried out by macrophages in the invading granulation tissue, organizing the blood clot. The subsequent events occurring in this granulation tissue may proceed in a variety of ways depending upon circumstances.

Thus, the granulation tissue may ossify or chondrify comparatively rapidly where the fracture has been adequately immobilised or it may become increasingly fibrous where mobility between fragments is excessive. This fibrous tissue may persist for a long time until sufficient immobility is provided by an adequate external callus after which it may then ossify directly or after prior conversion into fibro-cartilage. In exceptional cases, fibrous union and pseudoarthrosis may result.

B. Bone Formation in Fracture Repair.

The fibro-callus provides a collar of collagenous fibres around the area of the fracture and between the bone fragments. The portion of the callus which forms away from the fragment ends and extends along the unaffected bony surface has been

termed the periosteal callus (or anchoring callus). The endosteal callus is that portion which replaces the bone marrow. The uniting callus is found between the fragment ends. The voluminous part of the callus, i.e., that part which encircles the fracture area itself is called the bridging callus (Weinman and Sicher, 1955).

Ham and Leeson (1961) consider the cells of the cambium layer to be primarily responsible for the formation of the external callus, while the osteogenic cells of the endosteum and the marrow form the internal callus. The role of the 'fibroblastic' blastema and connective tissue 'induction' in contributing to osteogenesis has already been discussed in Chapter One.

Mineralization of the fibro-callus follows an orderly pattern, in the following sequence: the periosteal callus, the endosteal callus, the bridging callus and lastly the uniting callus. This has been confirmed both histologically (Urist and McLean, 1941, a & b; Ham and Harris, 1956) and micro-radiographically (Nilsonne, 1959).

In the periosteal callus, cancellous bone is formed, the trabeculae of which are more or less parallel and lie perpendicular to the bone surface. The endosteal callus is

formed by irregular woven bone which develops some distance from the fracture line and gradually extends up to the line of fracture.

The presence of cartilage, in particular, in the bridging callus, is a prominent feature. When calcification of the periosteal callus reaches the area of the bridging callus, replacement of the cartilage by bone ensues. When this replacement of the cartilage is completed, the entire fracture area is surrounded by a bony bridge, the primary bony callus.

The uniting callus which fills in not only the area between the fragments, but also the areas of bone resorption following the fracture, is the last portion of the callus to be replaced by bone.

Formation of the secondary bony callus occurs when the cartilagenous bridging callus is almost entirely replaced by immature bone and involves not only the replacement of immature bone by more highly calcified lamellated bone, but also the formation of compact bone in certain areas.

Pritchard (1964) distinguished three phases in new bone formation associated with the healing of a fracture. In the first phase (periosteal callus formation) osteogenesis is stimulated over a very wide area of the periosteum, involving

almost the whole surface of the injured bone, and he believes this to be a non-specific response to injury, related to inflammation.

In the second phase (bridging callus formation) a much larger and more localised mass of new bone, and perhaps cartilage as well, accumulates around the site of damage. The size of this mass is related in a general way to the amount of damage, but is more directly proportional to the functional weakness of the damaged bone, serving as a splint, until it has reached sufficient size and rigidity to immobilise the fracture.

In the third phase (uniting callus formation) the dead bone on either side of the fracture is resorbed and the gap is filled in with new bone. The redundant splinting bone is then resorbed.

There is considerable overlapping in the appearance and duration of these three phases of osteogenesis in fracture repair, all of which may be observed at the same time.

C. Cartilage Formation in Fracture Repair.

The appearance of cartilage in callus tissue is a common feature in the healing of fractures in long bones. Cartilage production has also been reported in the repair of

parietal bone fractures (Pritchard, 1946) and in bore-holes in cortical bone (Melcher and Irving, 1962; Radden, 1964).

The conditions giving rise to cartilage, instead of bone, in skeletal repair are not known. The amount of cartilage formed depends on species (Pritchard and Ruzicka, 1950), age, amount of damage to the bone and the mobility of the fracture (pritchard, 1964). Cartilage production by osteogenetic cells has been attributed to relative ischaemia (Ham, 1930; Girgis and Pritchard, 1958) and to the presence of pressure and shearing forces at an unstable fracture line (Weinman and Sicher, 1955; Pritchard, 1964). Cohen and Lacroix (1955) stated that the more intense the proliferation of cells, the more likely cartilage is to form.

Bassett (1962) from results of tissue-culture experiments, suggested that the pleuripotent mesenchymal cells are capable of differentiating into osteoblasts, chondroblasts or fibroblasts, depending on environmental factors. His experiments demonstrated that in the presence of compression, bone was formed when the oxygen tension was high, and cartilage was formed where the oxygen tension was low. In the presence of tension in the cell-mass and a high oxygen tension, fibrous tissue was produced.

Pritchard (1964) by comparing healing of immobilised and unstable fractures in the rat, pointed out the varying roles that the 'osteogenic' and 'fibroblastic' blastema play in these two situations.

In an immobilised fracture where the fracture gap is minimal and there is close apposition of the bony fragments the gap is bridged by osteogenic cells and union is achieved rapidly. In those fractures where the gap is wide, and apposition of bony fragments is prevented by mobility, the gap is colonized by fibroblasts and there is a temporary fibrous union; this is then followed by slow ossification of the fibrous tissue either by direct transformation of the fibrous tissue to bone, or after its prior transformation to fibro-cartilage.

Thus, Pritchard (1964) believed that two kinds of cartilage may make their appearance in the course of fracture repair:- hyaline cartilage, derived from the 'osteogenic blastema' and fibro-cartilage, derived from the 'fibroblastic blastema'. He pointed out that, "...furthermore, the orientation of collagen derived from the 'fibroblastic blastema' is dictated by the pattern of tensile and pressure stresses, and that fibrous tissue under pressure is converted to fibro-cartilage."

D. Quantitative Aspect of Callus Formation.

The varying roles that 'osteogenic' and 'fibroblastic' blastema play in the healing of immobilised and unstable fractures has already been pointed out. It should be stressed that the callus formed under these circumstances differs not only qualitatively, but also quantitatively.

It has been shown that the size of the external callus formed is in proportion to the degree of reduction and fixation of the fragment (Friedenberg and French, 1952; Yamagishi and Yoshimura, 1955; Bagby and Hanes, 1958; Schnek and Willenegger, 1967; Matzen, 1967; Kuntscher, 1967).

In the presence of intimate and accurate approximation of the fragments and complete stability, healing without radiological evidence of callus formation is possible. Schenk and Willenegger (1967) likened the healing in these instances to healing by "primary intention" of an incised wound in soft tissues, with subsequent minimal scar tissue formation. These authors regard the presence of callus formation as being parallel with healing in soft tissues by "secondary intention" with the formation of excessive scar tissue.

Pritchard (1964) is of the opinion that among the factors which control the course of bone repair, some at least are in the nature of 'feed-backs' initiated from the abnormal features of the injury. He regards the size of the callus around the injury to be related in this nature to the mechanical weakness and instability of the bone.

However, he emphasised that not every phenomenon of bone repair can be ascribed to mechanical determinants in the environment. Thus, he regarded the early widespread reaction of the periosteum to be inflammatory in origin. In this, he is supported by Kuntscher (1967) who went further to state that callus is formed only by inflammation, the cause of the inflammation being entirely unimportant.

Kuntscher (1967) stated, "By experimentally induced inflammation in the medullary cavity, a desired amount of callus can be produced ranging from the thinnest periosteal layer up to massive sclerosis seen in osteomyelitis. No callus formation can take place without inflammation. The callus formed following fracture or osteotomy is of chemical origin, brought about by breakdown products arisen at trauma or operative intervention owing to destruction and death of cells."

To substantiate his contention, Kuntscher (1967) introduced a sterile metallic wire into the medullary cavity of a long bone. The rusting wire brought about an extensive endosteal and periosteal callus formation. In his view, gross callus formation in an unstable fracture is the result of excessive inflammation due to constant mechanical trauma at the site of injury.

II. HEALING OF CORTICAL DEFECTS IN BONE

Whereas healing following fracture of a bone has been well documented, there have been few descriptions of repair of a defect in bone, apart from the investigations of Ely (1927), Bourne (1944), Pritchard (1956), Murray, Holden and Roschlau (1957), Melcher and Dreyer (1961, 1962), Melcher and Irving (1962, 1963) and Radden (1964).

The normal healing process in a penetrating defect of the cortex of the rat femur has been described by Melcher and Irving (1962, 1964). The following is a summary of their observations.

A. The Formation of Periosteal and Endosteal Callus

The formation of a haematoma, its organization and the subsequent development of fibro-callus took place as in the healing of a fracture. At the same time, cells of the cambium layer of the periosteum, extending from the margin of the defect, began to proliferate. These cells soon laid down new bone to form a ring of periosteal callus. The periosteal callus proliferated to the defect margin, but generally did not, at this stage, extend beyond it, although occasionally it grew a short distance over the wall of the defect.

The ring of periosteal callus was not even in distribution; the quantity of callus which developed on some aspects of the defect was frequently more extensive than on others. It was thought that this variation in development may be related to functional stresses (Melcher and Irving, 1962, 1964). Similar non-uniform development of the periosteal callus occurred in the healing of saw-cuts in rabbit tibiae (Bast, Sullivan and Geist, 1925).

Shortly after subperiosteal activity had begun, cells of the endosteum started to develop the endosteal callus. This extended for some distance along the medullary surface of the femur, but did not reach as far as its periosteal counterpart.

Proliferation of the endosteal callus, unlike that of the periosteal callus, did not halt at the margin of the defect but rapidly grew across, thereby isolating the medullary cavity from the wound. Callus then proliferated into the cortical defect, to within a short distance of the external surface of the femur.

No bone formation was observed to have arisen from either the cut surface of the cortical bone or from the fibrous granulation tissue within the defect (Melcher and Irving, 1962). Extensive necrosis of the bone in the wall of the defect did

not appear to occur, an observation similar to that made by Ely (1927).

Filling in of the defect by new bone was followed by renewed proliferation of the periosteal callus. This now grew over the external aspect of the defect, thus covering the endosteal callus.

B. Remodelling

Gradually the periosteal callus was remodelled and converted into compact bone, after which it was gradually removed by subperiosteal osteoclastic resorption.

Concurrent with the renewed proliferation and remodelling of the subperiosteal callus was the resorption of the newly deposited endosteal callus. Most of the bone in the medullary one-half to three quarters of the defect was removed in this process. However, the trabeculae lining the wall of the defect were not usually resorbed and served to secure the remaining callus to the defect walls.

At this stage, the old cortical bone and the remaining callus at the margin of the defect were remodelled, thereby eliminating the line of demarcation. Gradually, the callus in the defect was replaced by lamellar bone; this was a slow process and was still incomplete twelve months post-operatively.

C. The Role of the Periosteal and Endosteal Callus.

Most of the literature on the healing of fractures stresses the importance of the periosteal callus in re-establishing continuity of bone fragments, and assigns to the endosteal callus a subsidiary role in this function. Enneking (1948), on the other hand, maintains that healing is brought about primarily by proliferation of the endosteal callus and that the periosteal callus only bridges the fracture line after the endosteal callus has filled the fracture gap.

Melcher and Irving (1962) agree with this latter concept and stress the apparent inability of the periosteal callus to bridge the defect without a scaffolding of endosteal callus. These authors pointed to the marked contrast between the rapid proliferation of the periosteal callus in a healing fracture and its comparatively minor role in the repair of a cortical defect. They stated: "Possibly some stimuli to the development of subperiosteal callus are initiated by rupture of the periosteum, and are then progressively intensified by fracture of the bone shaft and displacement of fragments. Other stimuli may arise from functional weakening of the bone and these will also increase as the disability becomes more severe. On this premise, fracture of a long bone, particularly if

there is considerable displacement, will provide an enormous stimulus to the formation of subperiosteal callus. Conversely, stimulation from a circumscribed defect will not be very marked because loss of both function and continuity are comparatively mild."

Periosteal callus can be induced to attain proportions far in excess of those customarily developed in the healing of a defect, and may give rise to a protuberance which in favourable circumstances has been observed to persist for periods of time up to eighteen months (Melcher and Dreyer, 1962).

If the large haematoma which forms following the preparation of a defect in the femur was protected by a well-adapted fairly rigid concave shield, a bony protuberance developed (Pritchard, 1956). This protuberance was maintained only for as long as it was protected by the shield and when the latter was removed it was resorbed. The formation of a bony protuberance in similar circumstances was shown earlier by Murray, Holden and Roschlau (1957) who protected the haematoma after removing a portion of the ilium of a dog.

Melcher and Dreyer (1962) have also noted subperiosteal

bone formation occurring on the surface of the femur opposite a defect. These authors believed that it was possibly stimulated by the alteration of the direction of the forces which were normally transmitted through that part of the bone.

CHAPTER THREE

HEALING IN THE EXTRACTION WOUND

Early investigations into the healing of the alveolar socket wound were mainly concerned with establishing the histological sequence of tissue repair, the time required for bone regeneration, and the time the epithelium takes to cover the surface wound.

Although the main characteristics have been well established, observations related to the time and location of bone replacement varied because the experimental animals used were not uniform as to species, age, sex and diet; in some instances, detailed data was not given.

I. THE SEQUENCE OF REPAIR.

A. In Dogs

Euhler (1923) was the first to investigate the healing of extraction wounds in dogs histologically and radiographically, and established the following stages of repair: (1) haemorrhage, (2) coagulation, (3) thrombosis of the vessels of the alveolar wall, (4) organization of the fibrin clot, (5) proliferation of the epithelium over the surface of the wound, (6) resorption of the damaged bony tissues,

and (7) formation of new bone.

Schram (1929) reported that the epithelium grew over the socket in eight days and that the cancellous bone projected into the organised blood clot from the base and sides of the wound at this time. After forty-eight days, cancellous bone formation was complete and new bone projected above the resorbed margins of the old socket without material loss of either buccal or lingual alveolar socket walls.

Both the above investigators found the first histological evidence of bone formation at eight days post-operatively. This was also confirmed in H. Meyer's (1935) study. However, bone formation has been noted as early as 5 days after extraction (Claflin, 1936; Hubbell and Austin, 1941).

Claflin (1936) reported on dogs and on human autopsy materials. He noted that the extraction wound heals slower in the human. For example, a three-week-old human extraction wound was equivalent to a wound nine or ten days old in the dog, and a three-and-a-half-month-old extraction wound in man was equal to that of an eight-week-old wound in the dog.

B. In Rats

Huebsch et al. (1952) made a histological and radiographic study of the extraction wound in the lower first molar socket

of sixty-day old male Long-Evens strain rats. They found the first evidence of organisation of the blood clot at 23 hours post-operatively, as indicated by the ingrowth of a large number of capillaries in the region of the periodontal membrane remnants. The ingrowth of fibroblasts into the coagulum occurred three days post-operatively. New bone formation occurred in the adjacent marrow spaces, but had not yet extended into the alveolar socket wall.

The first indication of bone formation in the socket itself was seen on the fifth post-operative day. The first bone formation was built onto the original "alveolar bone" which showed delicate trabeculae of bone matrix extending from the old bone. There seemed little resorption of the original alveolar socket walls, except at the crest of the alveolar socket walls.

After ten days of healing, the epithelium had proliferated across and closed the wound. At this stage, immature, coarse fibrillar bone was growing into the socket from the walls of the alveolus. The granulation tissue in the socket also showed a more mature structure, with a definite orientation of the cells.

Thirteen days after the operation, the socket was almost

filled with new bone. The epithelium at this stage resembled that of the surrounding mucous membrane, with the rete pegs fairly well developed.

Twenty-five days after extraction, the entire socket was filled with young bone, and the epithelium consisted of a fully differentiated, stratified squamous epithelium showing keratinization.

Radiographically bone formation was evident on the sixteenth post-operative day.

C. In Rhesus Monkeys

Simpson (1960, 1961) carried out a very comprehensive investigation into the healing of extraction wounds in rhesus monkeys, comparing the effects of various surgical techniques.

Following forceps extraction, epithelization occurred between one to two weeks. The sockets were filled with new bone in four weeks and had reached a fairly stable condition at the end of eight weeks. Bone formation was almost entirely limited by a line joining the tips of the alveolar crests. Resorption of the labial alveolar plate was a constant feature, but the lingual plate was little

affected. The contour of the alveolar ridge was maintained by the new bone which filled the socket before appreciable resorption had occurred. At the end of eight weeks, the original socket outline was largely destroyed. Simpson also noted that the remnants of the periodontal membrane left on the socket walls after extraction of teeth invariably degenerates and so far as could be ascertained, took no part in the healing process. This confirmed earlier observations of Euhler (1923) and W. Meyer (1924).

Schram (1929) and Mangos (1941) had previously reported that resorption of the buccal and lingual crests in dogs and in humans was slight and Euhler (1923) had stated that osteoclastic activity ceased after four weeks. In Simpson's experiments, resorption of the buccal alveolar crest was a noticeable feature and continued for at least eight weeks, although in the later stages it appeared to be part of a remodelling process. However, the height of the alveolar ridge was maintained by the formation of new bone.

D. In Human

Amler, Johnson and Salman (1960) using biopsy material from normal individuals studied the healing process histologically and histochemically, up to fifty days after the

extraction of teeth. As a generalization, they suggested the following sequence in the healing of an alveolar socket:

(i) Clot formation fills the entire alveolar socket and contains at this stage no metachromatic ground substance, glycoprotein or alkaline phosphatase.

(ii) Granulation tissue arises first at the periphery of the socket, accompanied by a metachromatic ground substance, glycoprotein and alkaline phosphatase, two to three days after tooth extraction. It invades the centrally positioned blood clot and replaces it completely by the seventh day.

(iii) As to bone formation, by the seventh day, osteoid is evident at the base of the socket in an extremely high metachromatic ground substance bounded by osteoblasts laden with large amounts of alkaline phosphatase in the cytoplasm. Newly formed bone spicules attach directly to the old bone. Mineralization takes place and trabeculae are formed gradually filling at least two-thirds of the socket fundus by the thirty-eighth day. Radiographic examination shows visible changes in the socket at about the eighteenth day. Definition of the lamina dura is lost about the thirty-eighth day. The radiopacity increases until a peak is reached at about the hundredth day, when the socket content is nearly identical in density to the surrounding alveolar process.

(iv) Epithelialization is evident at the fourth day and

is associated with the presence of glycogen and glycoprotein. Fusion of epithelium was noted in some specimens at 24 days, although in certain specimens fusion had not yet taken place by the thirty-fifth day.

Several observations are of particular interest. Mangos (1941) had noted, in human biopsy and autopsy materials, osteoblastic activity at ten days, whereas Christopher (1942), also working on human materials, observed deposition of mineral salts at fourteen days. In Amler et al.'s investigation, uncalcified bone spicules (determined by the von Kossa Reaction) were evident at the seventh day. These were either isolated or attached to old spicules of bone where they were continuous at the peripheral portion of the socket. The formation of new bone by direct attachment to the old bone without preliminary resorption has previously been reported by Hubbell and Austin (1941) in the dog, and by Christopher (1942) in the human. This was also noted by Amler et al.

Mangos (1941) stated that "...the epithelium has proliferated completely across the wound in two weeks", and, further, that "in twenty-one days the epithelium had definitely healed across the granulation tissue." Deebach (1935) held that an average of seventeen days is required for complete epithelialization. In Amler et al's specimens, it was

observed that the epithelium does not grow over the surface of the extraction wound and reach its final stages of fusion until a minimum of twenty-four days. These latter investigators recognised the wide range in time for the fusion of the epithelium and suggested that local and systemic factors such as the diameter of the socket wound, age, laceration of the gingivae, presence of foreign bodies, and pre-existing infection might account for this diversity.

II. LOCAL FACTORS INFLUENCING THE HEALING OF THE EXTRACTION WOUND

Local factors in the healing of the extraction wound have also been investigated. W. Meyer (1924) studied histologically the influence of sutures, excision of the gingivae, foreign bodies and infection on the healing process in dogs. He found that suturing of the wound accelerated the healing in general, whereas excision of the gingivae, foreign bodies and infection delayed it.

Glickman et al (1947) noted delay in healing in the presence of retained root fragments, and mentioned the interesting manner in which the epithelium formed extensions from the surface to enclose the sequestrating fragments.

Smith (1958) concluded that the epithelium is the

primary agent involved in the elimination of retained root fragments and necrotic bone spicules. The process is accomplished by epithelial proliferation around the retained fragments thereby exteriorizing them. When the fragment to be sequestrated is in the deeper portion of the socket, epithelium proliferated downwards forming a tract to the surface. This opening remains patent until elimination of the non-vital tissue is completed.

It was noted that the retained fragments are surrounded by inflammatory cells and epithelial proliferation seems to extend in the direction where there is inflammatory infiltration. The extent to which the epithelium proliferates appears to be proportional to the concentration of the inflammatory infiltration. On this basis, Smith (1958) suggests the presence of a chemotropic substance in the area of inflammation which attracts epithelial growth.

Simpson (1960, 1961) compared the effects of various techniques in extracting teeth and their relation to wound healing. Wound healing, according to him, in surgical extractions progressed in a similar manner to healing observed in simple forceps extraction wounds, but the removal of the bony crest in surgical extraction limited the height to which bone regenerated in the socket.

When burs were used for the removal of bone, epithelialization took longer than with forceps extraction wounds (Simpson, 1960). This appears to be due to the continued sequestration of small bone fragments produced by the bur. The reaction of bone to high speed rotary instruments was also studied; heat injury to bone is less and the initial repair response faster, in the cuts produced with ultra-speed instruments with a water coolant.

Clinical observation shows that the alveolar crest is progressively resorbed after the extraction of teeth. It was argued that by reducing the crestal bone post-operatively, the volume of the blood clot is reduced, and therefore the time required for bony replacement in the socket might be shortened. Simpson (1961) however, showed that there is no evidence to indicate that this is so.

W. Meyer (1924) has stated that suturing of the extraction wound accelerated the healing process. Simpson (1961) however noted no difference in time of epithelial closure of the wound in sutured and un-sutured wounds. He also argued that sutures might prevent the escape of products of inflammation and thus might delay healing. He noted, however, that the inflammatory infiltration in the first few days is reduced.

CHAPTER FOUR

OBJECTS AND METHODOLOGY OF THE PRESENT INVESTIGATION

In a preliminary investigation, the author had noticed an unusual phenomenon in the healing of the alveolar socket in rats and in guinea-pigs. Apart from the new bone being formed within the alveolar socket, a marked proliferation of subperiosteal new bone was also noted on the buccal aspect of the alveolar socket wall.

This phenomenon had only been reported by Boyne (1962, 1963, 1966), Boyne and Kruger (1962) and Reynolds (1963). It appears unusual that this periosteal reaction had not been observed in the many previous investigations into post-extraction healing of alveolar bone.

Formation of periosteal callus is a pronounced feature in healing of fractures, and is also a consistent feature in healing of cortical defects in bone (Chapter 2). It was therefore suspected that periosteal callus formation may be a universal reaction of bone to trauma.

As far as is known to the author, no attempt has been made to correlate the reactions of injured bone following different types of trauma, for example, fracture, cortical

defects (such as drill-holes, saw cuts), and dental extraction. It seems desirable that such a comparison should be made so that the principles of bone repair may be better understood.

Apart from this, there are minor aspects in the healing of alveolar sockets about which there is diversity of opinion. The manner in which bone fills in the alveolar socket, and the role of the remnants of the periodontal ligament in repair are issues which require further elucidation.

In considering the healing of an incision in soft tissues, Dunphy and his associates have attempted to correlate the histological, chemical, histo-chemical and physical aspects into an integrated concept. Udupa and Prasard (1963) have provided a similar correlation in the healing of fractures. These authors and others (Chapter One) have stressed the importance of the role of the ground substance and, in particular, the mucopolysaccharides, in the early phases of wound healing.

Histochemical observations on ground-substance and intercellular fibre formation have therefore been made in this investigation.

Methods which will provide information on the following aspects of healing have therefore been used:-

(i) The activity of various cellular components taking part in the repair process,

(ii) the identification of ground substances, intercellular fibres and osseous matrix,

(iii) the identification of new bone formation.

These methods have been applied in order to follow the healing process after the extraction of a maxillary molar in the rat.

The theoretical basis of methods that have been used in the present investigation will now be discussed.

I. IDENTIFICATION OF CELL PROLIFERATION

The identification of cell proliferation by counts of mitotic division on routine histological sections require good fixation and staining. These are difficult to achieve. Often, identification is difficult because of the small size of the nuclei in some cells e.g. small lymphocytes. Further, owing to the loss of characteristic cytological features during the mitotic cycle, it is not possible to ascertain, in tissues containing a mixture of cell types, which cell type

is undergoing division (Leblond, Messier and Kopriwa, 1959).

In recent years, the use of specific radioactive labels in conjunction with autoradiography has produced rapid advances in the study of cell proliferation.

Nucleic acid precursors labelled with carbon (C^{14}) and tritium (H^3) have proved to be very useful, the most commonly used being tritiated-thymidine. Such tagging techniques have not been used in the present study and therefore will not be further discussed.

Less accurate and sophisticated methods of identifying dividing cells are also available. These enable a limited insight into mitotic activities and indirectly into tracing the cell types in active proliferation.

That the plant alkaloid, colchicine, is capable of arresting mitosis was first described by Pernice (1899) in the epithelial cells of the stomach and intestine of the dog (Eigsti et al. 1949).

Brues (1936) reported the arrest of mitosis by colchicine in regenerating liver and showed that this drug stops all nuclear division at metaphase, without affecting the rate at which these divisions occur. Sentein (1942a

and b; 1943a and b), studying the effect of colchicine on larval tissues and in the hypophysis of the guinea pig agreed that there was no stimulation of mitosis; colchicine acted merely to block mitotic division at metaphase.

It is believed that the main action of colchicine is in altering the properties of the spindle (Eigsti and Dunstin, 1955). The fibrous and polarized spindle is rapidly changed into an amorphous "pseudo-spindle" or "hyaline globule" which is incapable of moving the chromosomes (Gaulden and Carlson, J. 1951; Inoue, 1952). As a consequence, the chromosomes remain clumped together in the central part of the cell, thus making the affected cells readily recognizable in histological sections.

Leblond and Stevens (1948) found that 6 hours was the most suitable period of time for the optimal demonstration of the action of colchicine. In studies of cell renewal up to 6 hours following injection of the drug, there were very few anaphases and no telophases. It was concluded that the mitosis had not progressed beyond the metaphase stage.

Furthermore, after the 6 hour period, many of the colchicine metaphases were observed to undergo pyknosis and hence could become unrecognizable as such. Bullough (1949) reported

that if mouse tissues were examined at time intervals longer than 5 to 6 hours following colchicine administration, a decrease in the number of metaphases occurred. He attributed this to an inhibition of mitosis by colchicine after the 6-hour period. Storey and Leblond (1951), however, commented that this decrease in mitotic number may have been more apparent than real, since it was probable that some colchicine metaphases had undergone pyknosis and fragmentation and therefore become unrecognizable.

The work of Bertalanffy and Leblond (1953) showed that the optimum dose of colchicine for rats to be 0.1 mg. per 100 gm. of body weight. This dose, administered in a single injection arrested many divisions at the metaphase stage and prevented the accumulation of anaphases and telophases, and did not appear to affect the experimental animals adversely.

II. IDENTIFICATION OF GROUND SUBSTANCES AND INTERCELLULAR FIBRES.

A. Ground Substances

In this study, ground substances were demonstrated by the PAS stain and by Hale's colloidal iron stain (Appendix VII).

(a) Periodic Acid-Schiff's Reagent

Periodic acid is a selective oxidant that attacks the

following groups:- 1,2 glycol, 1-hydroxy-2-amino, 1-hydroxy-2-alkylamino, and 1-hydroxy-2-keto (Barka and Anderson, 1963). As a result of oxidation, at least one aldehyde group is formed, which is subsequently visualised by Schiff's reagent.

If glycogen is first removed by treating the tissues with Diastase, then a positive PAS reaction strongly indicates the presence of glycoproteins, mucoproteins and glycolipids (Barka and Anderson, 1963). Neutral mucopolysaccharides will also react with the PAS stain; however, these do not occur commonly in higher animals. Acid mucopolysaccharides play no significant role in PAS staining, these being either PAS-negative or give a weak reaction (Barka and Anderson, 1963).

(b) Hale's Colloidal Iron Stain

Various histochemical stains have been used to demonstrate the presence of acid mucopolysaccharides in tissues, with varying degree of specificity. These include the use of alcian blue, astra blue, toluidine blue and the colloidal iron method.

In this investigation, the colloidal iron absorption method of Hale has been used. Acid mucopolysaccharides are

the principal extra-nuclear substances which absorb colloidal ferric hydroxide. When sections are treated with an acidified colloidal solution of ferric hydroxide, acid mucopolysaccharides absorb the colloidal ferric hydroxide. Iron in the bound colloid is then demonstrated by the Prussian blue reaction. The intensity of staining seems to vary with the amount and degree of polymerisation of the mucopolysaccharides (Barka and Anderson, 1963).

Immers (1954) stated that this method is not specific for acid mucopolysaccharides, since phospholipids, polynucleotides, and phospho-proteins are also stained.

Since its introduction, a number of modifications have been advanced to further its specificity for acid mucopolysaccharides. The method of Mowry (Lillie, 1965) in the preparation of dialysed colloidal iron solution has been used (Appendix VII).

In this study, sections were stained with PAS (Appendix VII) and the positive areas were interpreted as indicating the presence of glycoproteins or mucoproteins. The next consecutive sections were then stained with Hale's colloidal iron method, which revealed the presence of acid mucopolysaccharides.

In some sections, a combined Hale and PAS staining procedure was used.

(c) Intercellular Fibres

Collagen fibres are the most important functional element in granulation tissue. Collagen fibres were demonstrated by the Van Gieson and Mallory's aniline blue stains in this study (Appendix VII).

Compared with collagen, reticulin fibres are much finer, of irregular course and widely anastomosed. They are not demonstrated by routine stains such as hematoxylin and eosin. Special stains, of which silver impregnation is the most widely used, give reticulin fibres a uniform black colour. Amler et al. (1964) have summarised the differences between collagen and reticulin fibres.

With additional techniques, at least three varieties of reticulin fibres have been distinguished; (i) pre-collagenic fibres which eventually become collagen, seen mainly in embryonal tissues and in wound healing (Robb-Smith, 1957), (ii) fibres which are apparently cytoplasmic prolongations of reticulum cells in the spleen and lymphoid organs (Lillie, 1952), and (iii) argyrophilic fibrils, which are

neither dependent on reticulum cells, nor do they mature into collagen fibres, such as those in the stroma of the kidney and liver (Kramer and Little, 1953).

In this study, a silver impregnation technique (Lillie, 1965) has been used (Appendix VII) to demonstrate pre-collagenic argyrophilic fibrils

III. IDENTIFICATION OF NEW BONE FORMATION

Modern methods of bone research particularly within the last two decades have filled in many gaps in bone physiology and pathology. Micro-radiography was applied to the study of compact bone by Engström (1946). By this method, the distribution of bone salts has been investigated (Amprino and Engström, 1952; Owen, 1956; Wallgren, 1957). Micro-radiographical investigations into the healing of fractures have been reported by Nilsonne (1959), and of bone-grafts by Holmstrand (1957) and in experimental jaw injuries by Omnell (1957).

Ca^{45} became available in quantities in 1948 and was widely used in the study of the osteon. In the field of fracture healing, experiments with various radioactive isotopes have revealed their rapid incorporation into the

newly formed bone tissue in the callus (Bohr and Sorensen, 1950; Cartier et al., 1956). Quantitative information on bone salt accretion in callus formation was also gained by this method (Bauer, 1954; Bauer and Carlsson, 1955).

These methods, that is micro-radiography and bone-seeking radio-isotopes, have not been used in the present study and will not be further discussed.

Since the discovery that madder root when fed to pigs stained the bone laid down during the same period of time, a number of agents have been found which behave similarly. The active principle of madder root, Alizarine Red S (ARS) has since been isolated and used in its pure form. The inhibitory effect on bone calcification by this agent has recently been reported (Harris, Travis, Friberg and Radin, 1964; Harris, Nagant De Deuxchaisnes, 1965). Other substances that stain new bone intra-vitally include trypan blue, tetracyclines, 2, 4-bis (N,N'dicarboxy-methyl-amino-methyl) fluorescein (DCAF) and chlorazol fast pink.

One of the tetracycline group of antibiotics and chlorazol fast pink have been used in the present study. These agents were chosen because of their complementary labelling mechanism; whereas the tetracyclines are bound

to the inorganic bone salts, chlorazol fast pink is incorporated in the organic matrix of bone.

A. The Tetracyclines

In the last few years it has been established that the tetracycline group of antibiotics are incorporated into bone at the sites where deposition of bone salts takes place if these drugs are present in the body fluids during this period. They remain fixed at these sites and presumably can only be removed in vivo by resorption of the involved portion of bone. The presence of these drugs in bone is easily detected by examination under ultra-violet light which causes yellow fluorescence of the tetracyclines.

The use of tetracyclines in bone labelling was reported by Milch et al. in 1957 and 1958. It was rapidly adopted by Lacroix et al. (1958), Ghosez (1959) and Ponlot (1960). Because of their bone-labelling properties, these drugs can be used as tracers to study bone growth, accretion and remodelling (Frost, 1961b; Gonin and Fleisch, 1962; Vanderhoeft, Kelly and Peterson, 1962; Amprino and Marotti, 1964; Sissons and Lee, 1964; Smeenk et al. 1965).

The mechanism of fixation of tetracyclines in bone is

not completely understood. Since the tetracyclines form chelates with calcium-ions and other di-valent cations (Regna et al. 1951) and as they are completely removed by decalcification (Milch, Tobie and Robinson, 1961) it appears that these drugs are bound to the inorganic component of bone. L. Meyers (1961) believed that the tetracycline molecule possesses the capacity of binding onto the surface of apatite crystals in vitro in a stereospecific manner, similar to the mechanism in the chemisorption of alizarin.

Fixation is mainly, though not completely, confined to growing surfaces. Fixation to apparently inactive surfaces (Frost et al. 1961, Harris, Jackson and Jowsey, 1962; Steendijk, 1964) and diffusion into bone deposited prior to administration of the drug (Ghosez, 1959; Frost et al. 1961; Harris, Jackson and Jowsey, 1962) have been observed.

Steendijk (1964) suggested that tetracyclines are fixed to bone by adsorption to the surfaces of bone salts. When the drug is present in the body fluids, it is adsorbed to every bone surface. On resting surfaces, it remains in a superficial position as long as the concentration in the body fluids is high enough, but it is "washed" off from these surfaces when the concentration in the surrounding fluids

falls. Within 48 hours after stopping administration of the drug, all optically detectable surface and diffuse staining disappears (Frost et al. 1961). On resorbing surfaces, it is dissolved with the bone salt on which it had been adsorbed. On mineralizing surfaces, it is incorporated into bone and cannot be "washed" off. Determination by both optical detection and by antibiotic diffusion in vitro (Bevelander, Nakahara and Rolle, 1959) seemed to indicate that a period of about 4 days after deposition in bone is required for the drug to be effectively immobilised.

This adsorption hypothesis, however, cannot entirely explain the staining of borders of osteocyte lacunae as has been observed by Frost et al (1961) and by Harris, Jackson and Jowsey (1962).

Steendijk (1964) stated that binding to the organic component of bone and cartilage plays no major role in the fixation of tetracyclines to bone. Soft-tissue binding has however been observed in mouse sarcoma (Loo, Titus and Rall, 1957), in malignant tumours of the gastro-intestinal tract (McLeay, 1958) and in mitochondria of cultured liver cells (Du Buy and Showacre, 1961).

In high doses, tetracyclines have been observed to inhibit

skeletal formation in the sand-dollar, in chick embryos, and in the calcification of regenerating teleost scales (Bevelander, Nakahara and Rolle, 1959; Bevelander and Joss, 1962; Rolle and Bevelander, 1966). Harris (1960) also found that high doses of tetracycline cause partial inhibition of mineralization in dogs.

In rats, a single intra-peritoneal injection of tetracycline, 20 mg. per kg. body weight, has not been found to cause any decreased calcification as compared with controls (Smeenk, Van Der Sluys Veer, Birkenhäger, and Van Der Heul, 1965). The labelling-time of such an injection was found to be 10 hours i.e. the bone was labelled during the 10 hour-period immediately following a single intra-peritoneal injection.

Of the tetracyclines, it has been found that demethychlor-tetracycline gives more intense fluorescence in ultraviolet light than other commercial preparations (Harris, 1960; Frost et al. 1961). Also relatively smaller doses of this anti-biotic may be used thereby minimizing possible harmful effects such as those reported by Bevelander and his associates and by Saxén (1966a and b).

B. Chlorazol Fast Pink

Whereas tetracyclines and many other bone labelling substances are bound to the mineral phase of bone, few agents are known that will label the organic matrix of forming bone. Moss (1954) reported that chlorazol fast pink, an acid disazo dye, was taken up by the matrix of newly formed bone. Weatherell and Hobbs (1959) agreed that this dye will stain the organic matrix of bone both in vivo and in vitro. They stated that the in vivo staining of the matrix by chlorazol fast pink does not appear to affect the growth of the tissue or its subsequent calcification, and therefore provides a means of labelling growing bone surfaces.

However, they concluded that the staining is not specific for newly formed bone, and that the dye is taken up where the matrix is decalcified and uncalcified (Weatherell and Hobbs, 1959).

Storey (1968), on the other hand, pointed out that histological study of thin undecalcified bone margins was not reported by Weatherell and Hobbs. Without this, it is difficult to localize the site of dye staining, because osteoid seams are no more than 4 to 5 microns wide and are undetectable in decalcified sections. By studying thin undecalcified

sections, and by examining the localization of the dye in connective tissues developed in turpentine induced abscesses and in strontium induced rickets, Storey showed that the dye is localized clearly at growing bone margins.

Storey stated, "This is demonstrated well by combined use of tetracycline and chlorazol fast pink which label the same areas of bone growth. That the two dyes are attached to different components of bone is shown by removal of tetracycline by EDTA demineralization, while chlorazol fast pink remains in previously calcified bone matrix."

Like other bone-seeking substances, chlorazol fast pink is retained within the bone structure as bone growth occurs and is removed when resorption of the stained areas occurs.

Some staining of connective tissue by chlorazol fast pink occurs. Elastic fibres are stained deeply, but collagen fibres only faintly so, and are virtually not detectable in thin sections. Macrophages also take up the dye (Storey, 1968).

The nature of the material that takes up the dye in elastic and bone tissues is not known. Storey (1968) believes that the intensity of staining is not related to either the collagen or mucopolysaccharide content of the tissues involved.

The main intensity of staining occurs within 24 hours of intra-vital injection. The main location of the dye is at calcifying bone margins, not in osteoid tissue. This is demonstrated by the fact that in rachitic rats, the wide osteoid seams were only faintly stained, but where calcification was still occurring, the colouration was intense (Storey, 1968).

Chlorazol fast pink is thought to be excreted largely by the kidney (Williams and Hodges, 1943). The localization of this dye in the proximal convoluted tubules may indicate re-absorption by the kidney. As Storey (1968) points out, such a process of re-absorption could explain a continual but decreasing intensity of staining of connective tissues as the dye is slowly lost from the body.

CHAPTER FIVE

MATERIALS AND METHODS

The present investigation involved the histological study of healing alveolar sockets in experimental animals at various post-operative intervals.

The ideal experimental animal is the rhesus monkey. This animal is phylogenetically close to man and has a dentition that is very similar to that of the human. Unfortunately, rhesus monkeys are not readily available and are costly.

I. CHOICE OF EXPERIMENTAL ANIMALS

A hooded mutant strain of the Rattus norwegicus has been used exclusively. The root form of rat molar teeth and the structure of their periodontium closely resembles that of human beings (Schour and Massler, 1949).

A view of the skull of the rat is shown in Appendix I (Fig. App.I.1.). The mandibular molars are not readily accessible even when tongue and cheek have been retracted, and vision is poor in these sites. For ease of access and manipulation, the upper first molar was chosen as the most

suitable for removal. It has the disadvantage of having five roots (Fig. App.I.2). The distal root sockets provide a histological picture resembling the sockets of a human molar when the sections are cut coronally.

Only young adult male rats weighing between 150 to 200 gms. were used. Male animals were preferred since the oestrous cycle may influence the pattern of wound healing, also, the mitotic activity of female tissues may also be affected similarly (Bullough, 1950, Ebling, 1954).

In a pilot study, it has been noted that with older and larger animals, (say 250 gms upwards), the formation of cementum on the root surfaces greatly increased the root length. This together with the increased bone-density in older animals, made the extraction of teeth without root fractures very difficult.

Also, in the older age groups, periodontal disease was often evident. Impaction of hair and food debris in the interdental papillae was common, and led to inflammation in these areas. As this would mask the histologic picture of inflammation and repair following tooth extraction, the use of older animals was avoided.

The choice of young adult animals was made with the

understanding that growth in these animals was still active and that results may not be strictly applied to animals of all age groups. Also, new bone laid down as part of normal growth had to be distinguished from that laid down through repair.

Animals were fed on a stock pellet diet (Appendix II) and water ad libitum, except for the immediate 24 hours post-operative period, when the animals were maintained on a bread and milk diet to avoid trauma to the oral wound.

II. OPERATIVE PROCEDURES

Animals were anaesthetised with a single dose of veterinary Nembutal sodium, 4.5 mgm. per 100 gm. of body weight, injected intra-peritoneally.

The animal was mounted on a special stand which prevented movement and which also provided traction to maintain jaw opening. After inspection for impaction of debris around the upper molar area, this region was cleansed with a 5% aqueous solution of chlorhexidine gluconate.

The gingival attachment of the upper left first molar was separated from the tooth surface with a fine sharp probe, (Fig. App. III.2). With a modified small dental spoon

excavator, and equipment as described in Appendix III, this tooth was approached from the palatal aspect, luxated and then removed (Fig. App.III.3). When there was excessive trauma induced through manipulation, or when a root fracture occurred, this was taken note of. In extractions where a fracture of roots occurred, the contra-lateral upper first molar was also removed. In a number of animals, fracture of roots occurred on both sides.

Following the removal of a tooth, one 5.0 braided black silk suture was passed with an atraumatic needle to approximate the edges of the socket wound. The animal was then returned to its cage for recovery.

III. EXPERIMENTAL PROCEDURES

Two series of experiments were performed post-operatively on these animals. The first series involved the intra-vital staining of new bone with demethy-chlor-tetracycline and chlorazol fast pink. This required the use of ultra-violet fluorescence microscopy to demonstrate areas of tetracycline labelled bone in undecalcified sections.

The second series involved the administration of colchicine to arrest mitotic activities and subsequent processing of specimens using routine histological methods. Both routine

histological stains and histochemical stains were applied to the study of tissue sections in this series of experiments.

A. First Series -- Intra-vital Staining of New Bone

Animals were injected intra-peritoneally with a combined dose of demethyl-chlor-tetracycline at a dose level of 50 mg. per kg. body weight and chlorazol fast pink at a dosage level of 25 mg. per 100 gm. body weight. Appendix IV gives details of preparation of these drugs. Injections were given daily for two consecutive days. A labelling time of at least 24 hours was allowed prior to sacrifice of the animal. Table 5.1 gives the schedule of injections given for each experimental group.

Animals were sacrificed at arbitrary post-operative intervals (Table 5.1) with an overdose of ether. Following sacrifice where applicable the upper right first molar was extracted. This served as the control for the experimental extraction site on the left side, and gave an estimation of new bone formed as part of the normal growth pattern during the experimental period. The extraction of this tooth was necessary, because the enamel of the tooth is too hard for sectioning un-decalcified.

Following the extraction of the control side molar, the entire maxillary complex was dissected free. Specimens were

fixed in 10% buffered formol-saline for at least 24 hours. Tissues were embedded undecalcified in a polyester resin and sectioned on an MSE sledge microtome at 7 to 9 microns thick. Sections were mounted in a polystyrene mixture. Appendix VI gives details of polyester resin embedding and sectioning according to the method of Ueckert (1960).

Serial sections were made of the entire alveolar socket area. Sections were mounted unstained and examined under transmitted ultra-violet illumination (Appendix V). The reproduction of fluorescence photo-micrographs is presented in Appendix V.

Consecutive sections were also stained with Van Gieson's Mallory's aniline blue and H & E stains. These sections provided a familiar histological picture for comparison, but will not give fluorescence under ultra-violet light microscopy. With these stains, sections were first decalcified in a 10% aqueous EDTA solution for 20 minutes before staining, as the acidity of these stains caused unsatisfactory staining in areas where calcium salts were present in the undecalcified sections.

B. Second Series -- Demonstration of Mitotic Activities

Animals were injected with a single sub-cutaneous dose of

colchicine (Appendix IV) at a dose level of 1 mg. per kg. of body weight, 6 hours prior to sacrifice. Animals were sacrificed with ether at arbitrary intervals (Table 5.2).

At sacrifice the maxillary complex was dissected free and fixed in 10% buffered formol-saline for a minimum of 24 hours. These were then decalcified in formic-formate solution, changing every 2 days until decalcification was complete (Appendix VII). These specimens were then embedded in wax using a double-embedding technique (Appendix VII). Serial sections covering the entire alveolar socket area were cut on a MSE rotary microtome at 7 microns.

Both routine histological and histochemical stains (Appendix VIII) were applied to these sections and examined under light microscopy.

TABLE 5.1

Intra-vital Staining with Demethyl-chlor-tetracycline and Chlorazol fast pink

Experimental Period	No. of Animals	Injection schedule (days post-operatively)
3 days	4	1st-2nd
5 days	4	3rd-4th
7 days	4	5th-6th
14 days	4	7th-8th
4 weeks	4	7th-8th, 19th-20th
8 weeks	4	7th-8th, 19th-20th, 33rd-34th
4 months	4	7th-8th, 19th-20th, 33rd-34th
8 months	4	7th-8th, 19th-20th, 33rd-34th

Total number of animals - 32

TABLE 5.2

Demonstration of Mitotic Activities with Colchine

Experimental Period	Number of Animals
4 hours	6
24 hours	6
48 hours	6
3 days	6
5 days	6
7 days	6
10 days	6
2 weeks	6
3 weeks	6
4 weeks	6

Total number of animals - 60

CHAPTER SIX

RESULTS

I. INTERPRETATION OF OBSERVATIONS

A. Arrest of Mitotic Divisions

In order to determine the effectiveness of colchicine an examination was made of the basal layer of cells in the oral epithelium not affected by trauma in each of the animals studied. Fig. 6.1 shows an example of the prominent mitotic divisions seen in the oral epithelium in the mid-palatal area.

The identification of mitotic figures in connective tissues was more difficult; particularly in areas where there were a mixture of cell types. Mitotic figures were claimed to have been identified only in cells in which the chromatic material had clumped together and presented with an irregular outline.

B. Demethyl-chlortetracycline (Tetracycline) Labelling

In appositional growth, tetracycline labelling of new bone produced distinct regular bands which gave a yellow fluorescence (Fig. 6.2). In areas of rapid proliferation the bands were less distinct (Fig. 6.3). In areas where the pattern of bone growth was multi-directional, for example, within the alveolar socket distinct bands were not formed and the configuration arising from tetracycline labelling was complex (Fig. 6.4).

It has been pointed out in Chapter 4 that fixation of tetracycline is mainly, but not completely, confined to growing surfaces. Diffusion into bone deposited prior to administration of the drug (Fig. 6.3), and fixation to the walls of osteocyte lacunae occurred. These points have been taken into account in the interpretation of results.

As young rats have been used, labelling of bone laid down as part of the normal growth pattern was inevitable. Examination of the control side socket gives an indication of the extent to which normal bone growth has taken place. Fig. 6.5 gives a comparison of the control side socket with the extraction socket in a 8 week old specimen (Fig. 6.6). It can be seen that during this period, the amount of bone laid down as part of the growth pattern was considerably less than that laid down in the region of osseous repair.

As a result of difficulties encountered in polyester resin embedding and sectioning of undecalcified tissues, some materials were rendered unsuitable for histological observation. This was particularly so with bone of the maxillary complex, where shattering of tissues on sectioning was a problem.

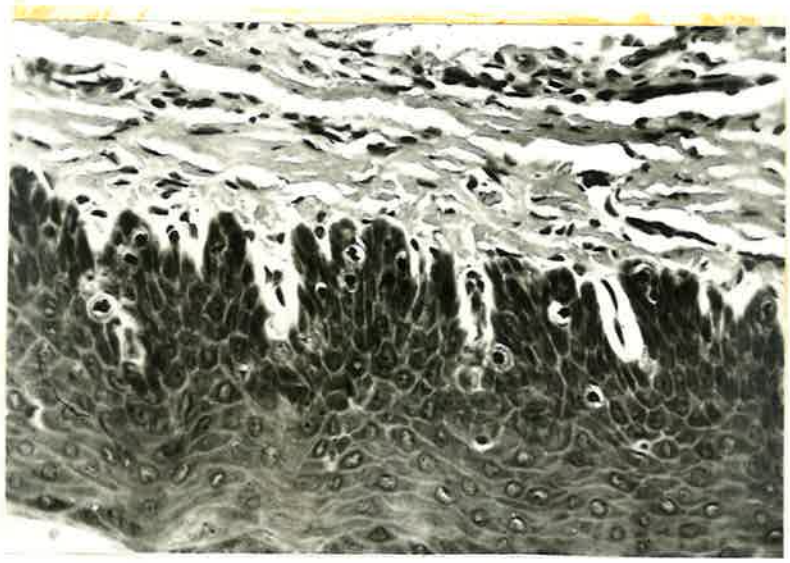


Fig. 6.1 Mitotic Figures in Oral Epithelium 6 hours following the administration of Colchicine

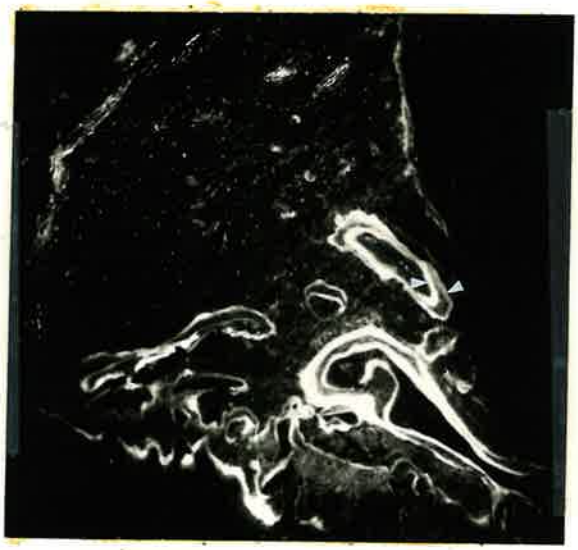


Fig. 6.2 Distinct bands of Tetracycline Fluorescence demonstrating Appositional growth on Endosteal Bone Surface

Fig. 6.3

Rapid proliferation of Bone on Periosteal Surface

Fluorescent bands are indistinct

Some diffusion into bone laid down prior to labelling is also seen (arrow)

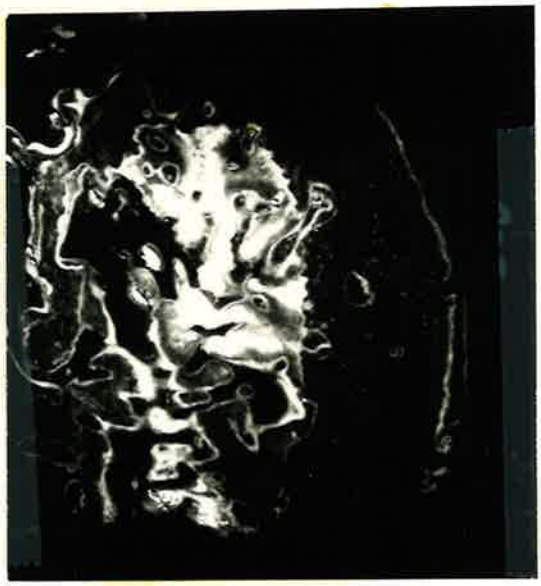


Fig. 6.4

Complex pattern of Bone formation in the Socket

Fig. 6.5

Control side 9 weeks showing three regular fluorescence bands on buccal cortical bone labelled at two weeks intervals.

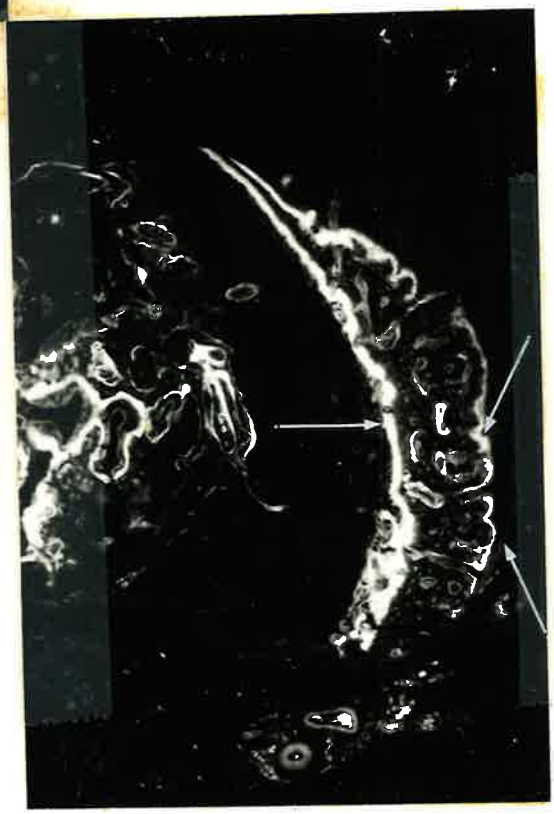


Fig. 6.6

Experimental side 9 weeks showing gross bone formation during the same period

HEALING IN THE EXTRACTION SOCKET

4 Hours

The socket was filled with a blood clot (Fig. 6.7) The remnants of the periodontal fibres were clearly visible (Figs. 6.8, 6.9). The blood vessels in this area and in the adjacent bone showed margination of polymorphonuclear leukocytes (polymorphs). These cells had also started to migrate into the periodontal remnants and into the margins of the blood clot (Fig. 6.9).

A fibrinous exudate, which was PAS positive, had also accumulated under the margins of the epithelium where there was also a concentration of polymorphs (Fig. 6.10).

At this stage, there was no sign of activity in the cambium layer of cells in the periosteum covering the buccal, palatal and nasal surfaces of the maxilla. Similarly, the cells of the endosteum lining the adjacent Haversian systems and bone marrow spaces were inactive. In one specimen, however, the fibrous layer of the buccal periosteum was haemorrhagic and an early inflammatory reaction was noticeable (Figs. 6.11, 6.12).

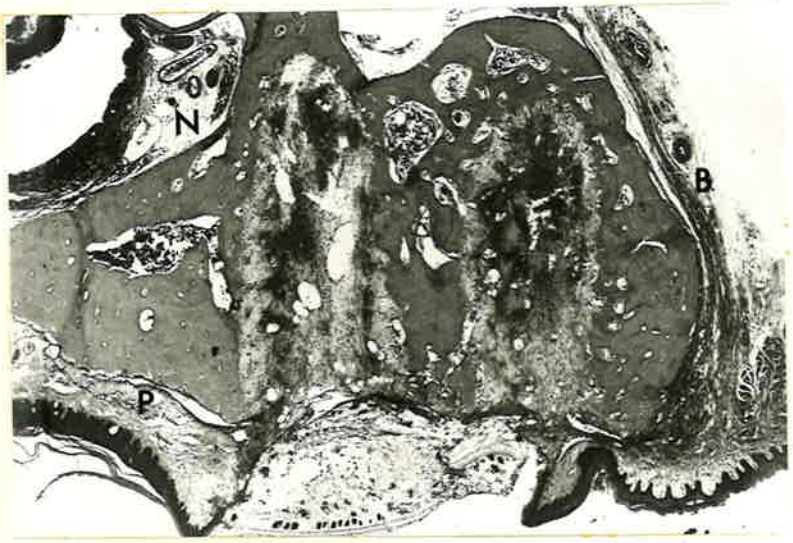


Fig. 6.7 H&E X 25. 4 Hrs. (Col.4)

Buccal surface (B), palatal surface (P), nasal surface (N).

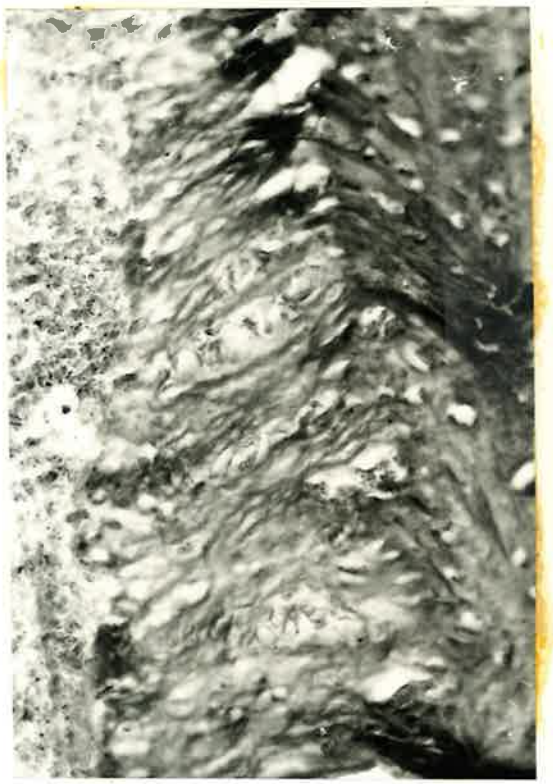


Fig. 6.8

VG X 250
4 Hrs. (Col.1)

Remnants of periodontal fibres

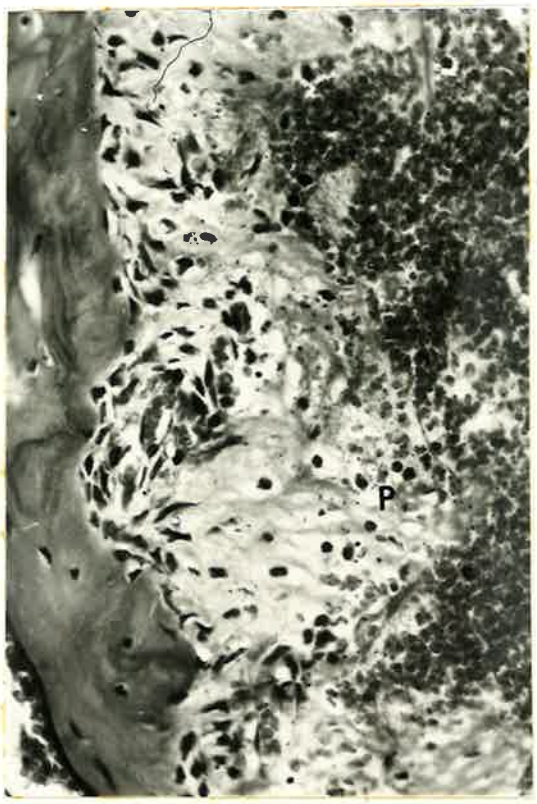
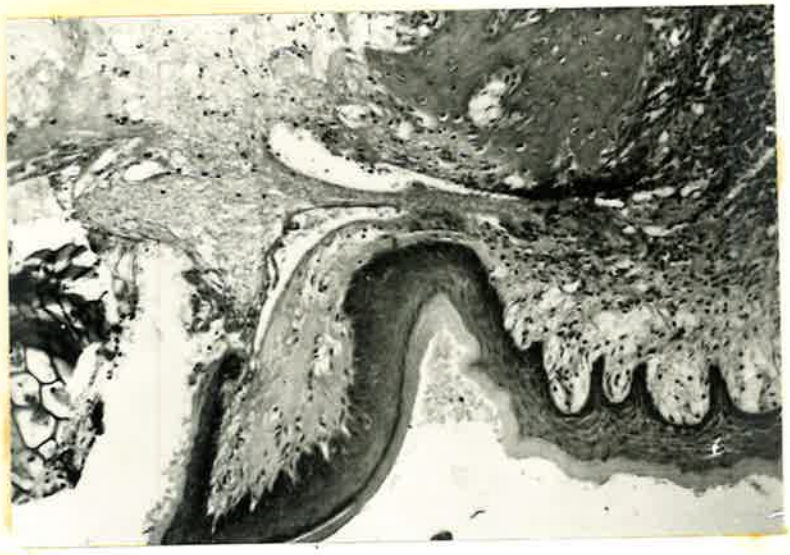


Fig. 6.9 H&E X 250
4 Hrs. (Col.4)

Remnants of periodontal membrane and blood clot. Early inflammation. Polymorphs (P)

Fig. 6.10 PAS X 100
4 Hrs. (Col.4)

Fibrinous exudate under margin of oral epithelium, with infiltration of inflammatory cells.



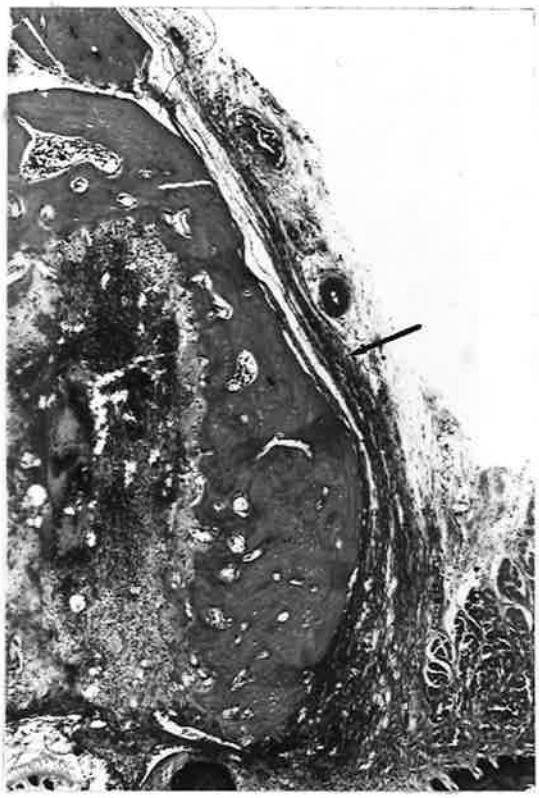


Fig. 6.11 H & E X 40
4 Hrs. (Col.4)

Early inflammation in extraperiosteal tissues. Periodontal remnants (arrow)

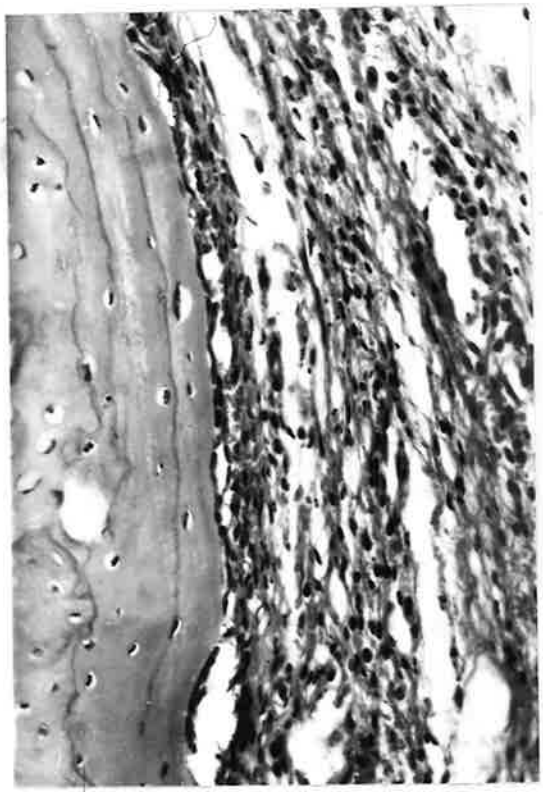


Fig. 6.12 H & E X 250
4 Hrs. (Col.4)

High power magnification of Fig. 6.11

24 hours

Lysis of the red blood cells had begun. This was particularly so in the superficial one third of the socket and around the margins of the blood clot (Fig. 6.13), leaving a network of PAS positive fibrin. The accumulation of PAS positive fibrinous exudate was most evident around the margins of the clot (Fig. 6.13) and particularly in areas close to capillaries (Fig. 6.14).

The remnants of the periodontal fibres were still clearly visible and no mitotic figures were noted amongst the "fibrocytes" in this tissue. Inflammatory cell infiltration into the periodontal remnant and into the marginal areas of the blood clot was by now a prominent feature.

In some specimens, the buccal periosteal tissues were edematous and infiltrated with inflammatory cells (Figs. 6.16, 6.17). The cambium layer of cells showed plump and dark staining nuclei (Figs. 6.16, 6.17), compared with the cells of the buccal periosteum on the control side (Fig. 6.15). Active mitotic division in this layer of cells was, however, not seen at this stage.

The first appearance of osteoclasts was seen and these were in the fundus region of the socket (Fig. 6.18).

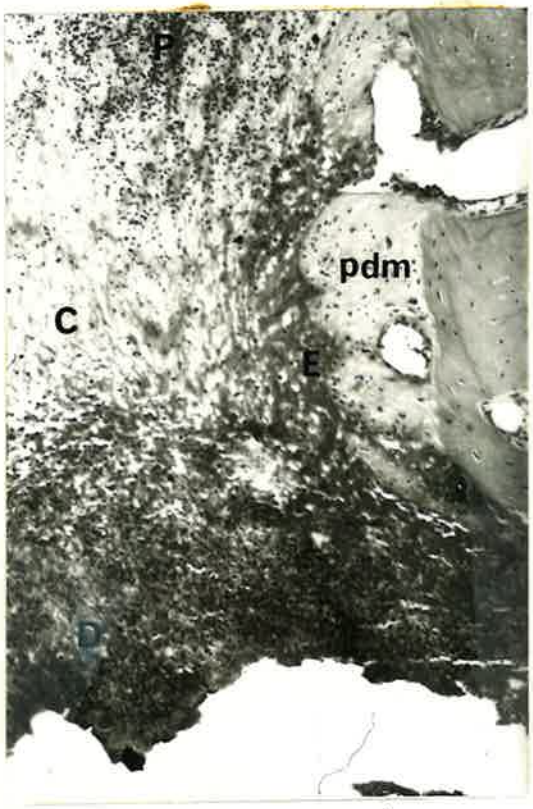


Fig. 6.13 PAS X 100
24 Hrs.(Col.6)

Lysis of blood clot and infiltration of polymorphs in superficial portion of blood clot.

Debris (D), Lysed blood clot (C), polymorphs (P), periodontal remnants (Pdm), fibrinous exudate (E).

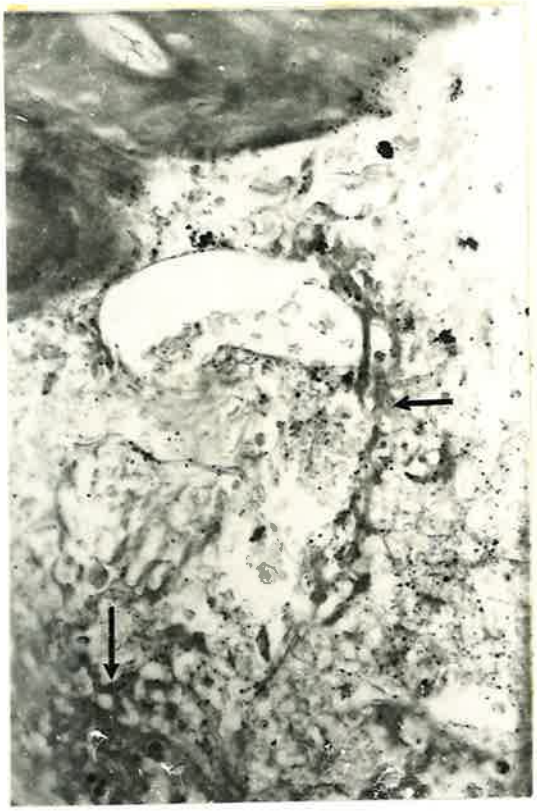


Fig. 6.14 PAS (no counter stain) X 250
24 Hrs. (Col.5)

PAS positive material around capillaries (arrows)

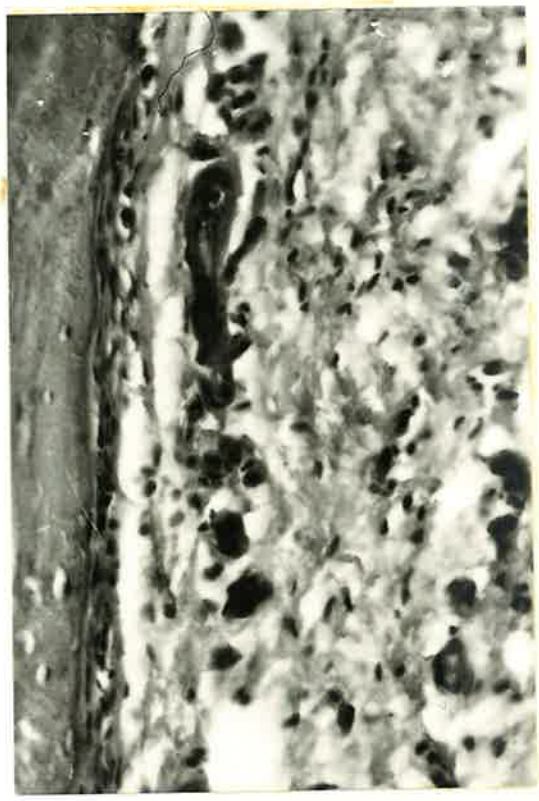


Fig. 6.15 H&E X 250
24 Hrs. (Col.3)

Normal periosteum on buccal surface of alveolus on control side

Fig. 6.16 H&E X 250
24 Hrs. (Col.3)

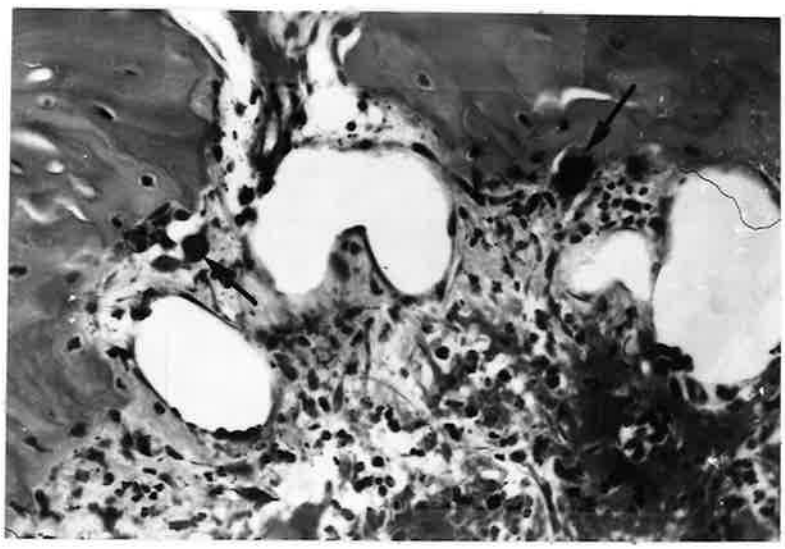
Edema and inflammation in buccal periosteal tissue.

Fig. 6.17 H&E X 250
24 Hrs. (Col.6)

Edema and inflammation in buccal periosteum, but less intense than that seen in Fig. 6.16

Fig. 6.18 H&E X 250
24 Hrs. (Col.6)

Appearance of osteoclasts in fundus region of socket (arrows)



48 Hours to 72 Hours

At 48 hours, the remnants of the periodontal fibres were less distinct and were staining less strongly with Van Gieson's stain than in the 24 hours specimen (Fig. 6.10). They now contained many small blood vessels, particularly in the fundus region (Fig. 6.21). By 72 hours, the degeneration of the periodontal fibres was more evident (Fig. 6.20).

The blood clot was in an advanced state of lysis at 48 hours, and the first cells recognisable as fibroblasts had infiltrated into the lysed blood clot at its margins. These were stellate in shape and were most abundant around small blood vessels close to the walls of the alveolar socket (Fig. 6.23). Owing to the early appearance of granulation tissue and the mixture of cell types in the remnants of the periodontal membrane, it was difficult to determine to what extent the "fibrocytes" in this tissue had participated in the formation of granulation tissue.

The accumulation of PAS positive material was particularly noticeable at the margins of the blood clot, and close to the invading capillaries (Figs. 6.21, 6.22).

At 72 hours, a faint Hale's staining reaction showed the

presence of acid mucopolysaccharide in the interstitial areas in the granulation tissue (Fig. 6.24).

At 48 hours, osteoclastic resorption was increased, but was still mainly confined to the fundus region of the socket. By 72 hours, intense osteoclastic activity was noted in some specimens, but much less so in others. Osteoclastic resorption had also extended along the walls of the socket (Fig. 6.25).

The cambium layer of cells in the buccal periosteum showed mitosis at 48 hours in some specimens. On the periosteal and endosteal surfaces in other locations, there was no such change. By 72 hours, subperiosteal "osteoid" was noted in some specimens on the buccal surface of the alveolar process. The active periosteum stained moderately with PAS in the interstitial areas around the osteoblasts (Fig. 6.26). Acid mucopolysaccharide had also started to accumulate in the edematous zone outside the active cambium layer.

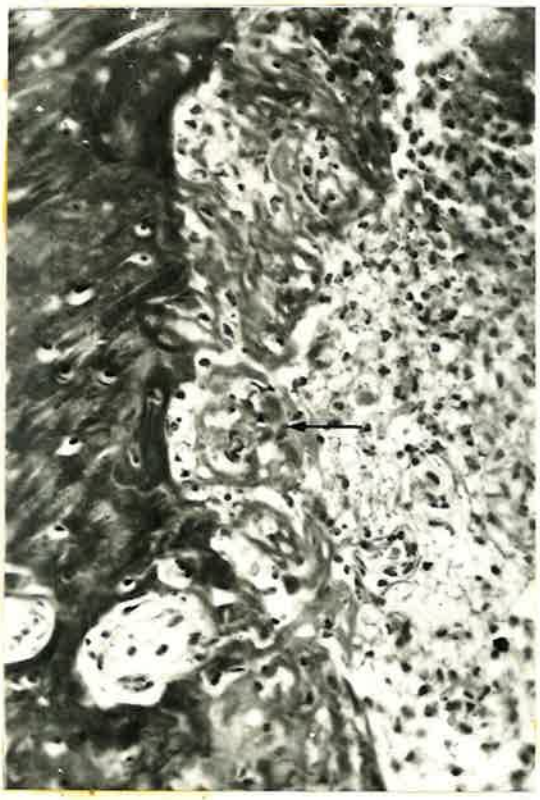


Fig. 6.19 VG X 250
48 Hrs. (Col.1)

Degeneration of periodontal fibres (arrow)

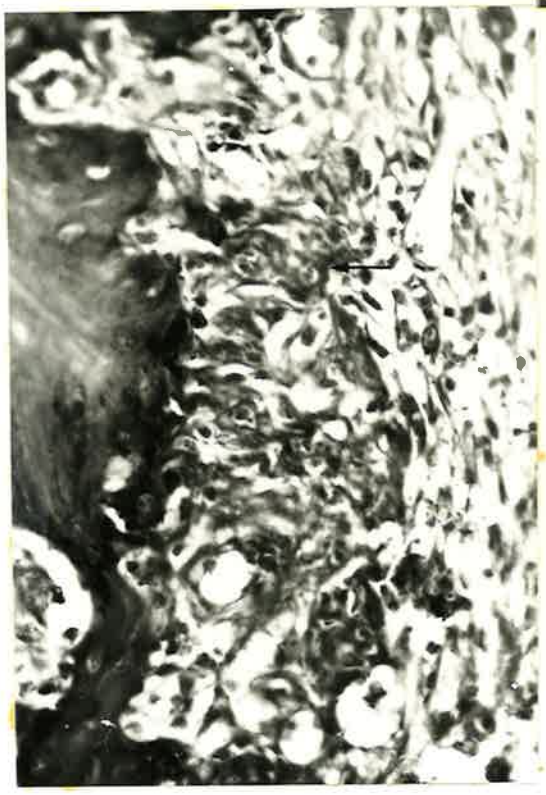


Fig. 6.20 VG X 250
72 Hrs. (Col.1)

Further degeneration of periodontal fibres (arrow)

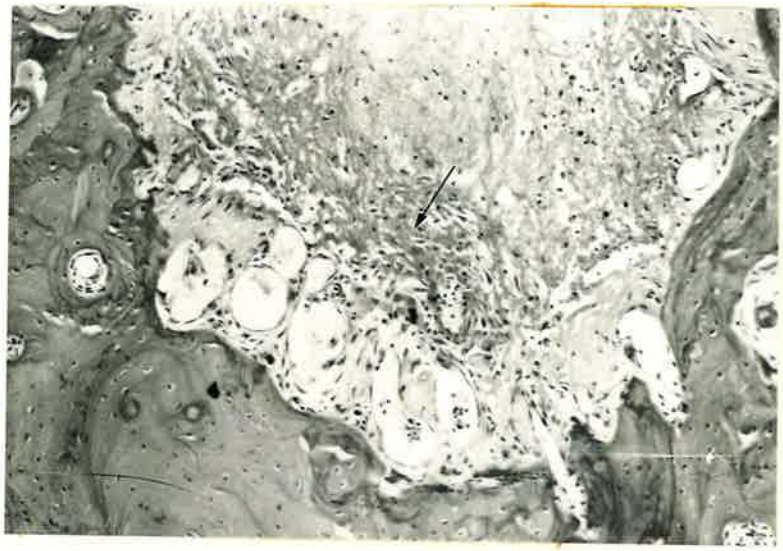


Fig. 6.21 PAS X 100 48 Hrs. (Col.1)

Thin walled vessels in periodontal remnants. Accumulation of PAS +ve material around blood vessels (arrow)

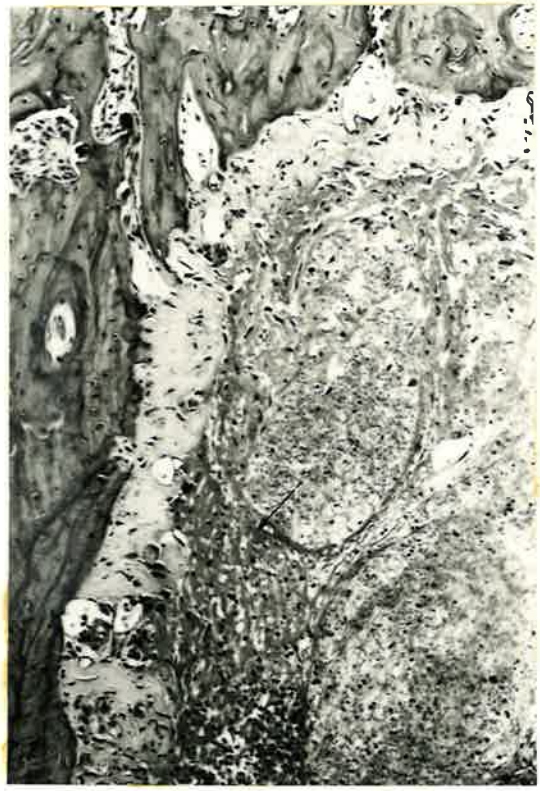


Fig. 6.22

PAS X 100
48 Hrs. (Col.2)

Fibrin network and PAS +ve material (arrow)

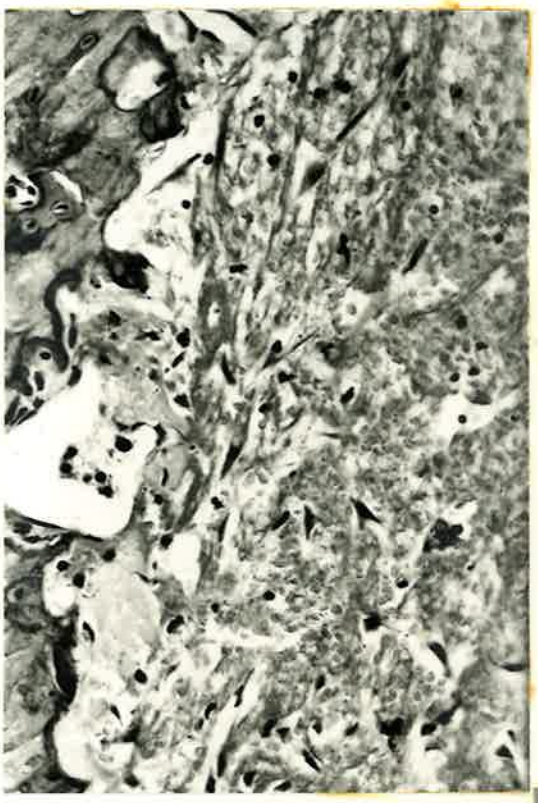


Fig. 6.23 H&E X 250
48 Hrs. (Col.4)

Stellate shaped fibroblasts in early granulation tissue

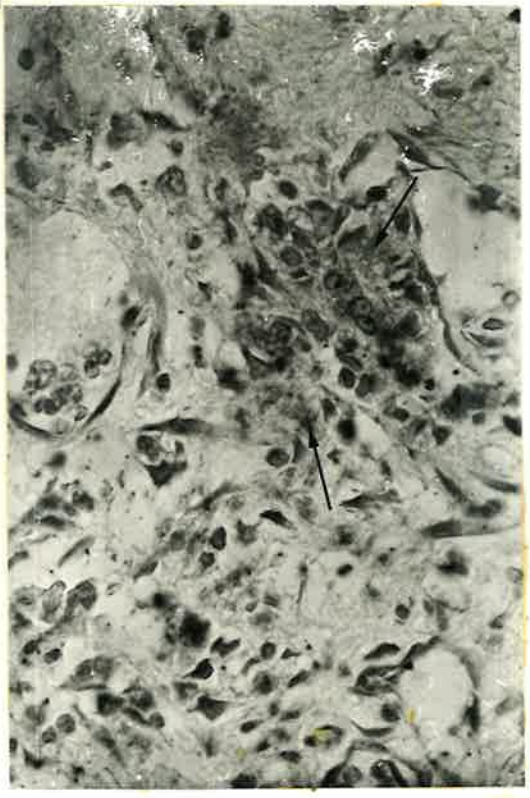


Fig. 6.24 Hale X 400
72 Hrs. (Col.3)

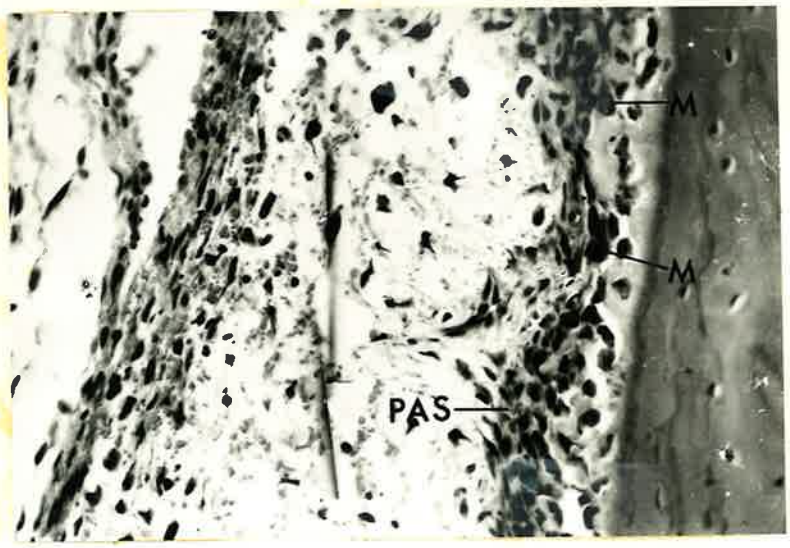
Acid mucopolysaccharide (arrows) in interstitial areas of cells in granulation tissue

Fig. 6.25 H&E X 100
72 Hrs. (Col.1)

Osteoclasts (arrows)in fundus regions and side of socket

Fig. 6.26 PAS X 100
72 Hrs. (Col.1)

Active cambium cells and early osteoid on buccal periosteal surface
Mitosis (M), PAS +ve Material (PAS)



5 days

On the fifth day post-operative, the socket was filled to two-thirds by granulation tissue. From the superficial surface of the socket to the fundus, five distinct zones could be distinguished (Fig. 6.27):-

(i) A layer of necrotic material covering the superficial surface of the socket.

(ii) A layer of acute inflammatory cells, which contained a large number of necrotic cells and exudate, which separated the debris from the underlying tissues,

(iii) A layer of mesh like fibrin, which was infiltrated with polymorphs, macrophages and round cells,

(iv) Granulation tissue, with many spindle-shaped fibroblasts and infiltrated with polymorphs, macrophages and round cells. This layer also contained many small thin walled capillaries (Fig. 6.28).

(v) New trabecular bone in the fundus region of the socket.

The granulation tissue contained many spindle-shaped fibroblasts and many mitotic figures were seen around capillaries (Fig. 6.30). The intercellular spaces in the granulation tissue stained positively with PAS (Fig. 6.29) but much less strongly than in earlier stages. Hale's stain now gave a weak and

diffused positive staining reaction of the intercellular substances. Silver staining, showed a large number of argyrophilic fibres, arising perpendicular to one bone surface and then ramifying in the granulation tissue (Fig. 6.31). Van Gieson's stain also stained the intercellular fibres weakly (Fig. 6.32). These fibres corresponded closely in position to the distribution of the strongly argyrophilic fibres. By this time, the remnants of the periodontal fibres were staining very weakly with Van Gieson's stain. This tissue component had lost its fibriller structure, and appeared homogenous and could only be seen in isolated areas (Fig. 6.32).

In some specimens, osteoclastic resorption was very active and had been responsible for the resorption of a large portion of the socket walls and interseptal bone (Figs. 6.33, 6.34, 6.35). In these specimens, food impaction into the socket was present and an intense inflammatory reaction was evident around this foreign material. The formation of granulation tissues was far less advanced in these specimens.

The first sign of new bone formation in the granulation tissue was apparent. This occurred in the fundus region of the socket (Figs. 6.36, 6.37, 6.38). The new bone was trabecular (Fig. 6.36, 6.38) and was lined by active osteoblasts, amongst the interstitial areas of which weakly staining

acid mucopolysaccharide (Fig. 6.41) and moderate PAS positive material (Fig. 6.40) could be demonstrated. Many mitotic figures were seen amongst these osteoblasts (Fig. 6.39).

Fig. 6.42 shows chlorazol fast pink staining and Fig. 6.43 shows tetracycline staining of new bone in the socket. A comparison between these two figures shows that chlorazol fast pink had stained regions which had not taken up tetracycline, indicating that these regions had not been mineralized. Regions which had taken up tetracycline staining were only lightly stained with chlorazol fast pink.

At this time, new bone was continuously being laid down on the buccal surface of the alveolus. Many mitotic figures were seen in the cells of the periosteum (Fig. 6.44). This bone was also trabecular (Figs. 6.45, 6.46) and was laid down perpendicularly to the cortical surface. In between the periosteal osteoblasts there was now an accumulation of strongly PAS positive material (Fig. 6.47). A build up of acid mucopolysaccharides in the zone outside the osteoblasts was also evident (Fig. 6.48). Figs. 6.49 and 6.50 show calcified and uncalcified portions of the new periosteal bone as demonstrated with chlorazol fast pink and tetracycline staining respectively.

Silver staining showed an abundance of argyrophilic fibres

arising from the surface of the trabecular periosteal bone. These ramified between the osteoblasts (Fig. 6.51). Van Gieson's stain also demonstrated the presences of fibres arising from the trabecular bone surface (Fig. 6.52). The amount of new bone formed subperiosteally was not uniform in all specimens. In sockets where there had been food impaction and widespread resorption of the socket, the amount of new periosteal bone formed was more extensive (Fig. 6.33, 6.34, 6.37) than in sockets where resorption had been minimal and healing in the socket was advanced (Fig. 6.36). In the former specimens, inflammation in the buccal tissues outside the periosteum was also more pronounced (Figs. 6.33, 6.34, 6.37). In areas were inflammation was intense, or where the buccal epithelium had been displaced and the cortical bone exposed, there was no new periosteal bone formed (Figs. 6.33, 6.34, 6.37).

Fig. 6.27 H&E X 40
5 Days (Col.4)

Palatal socket showing five zones.

1. Debris
2. Inflammation
3. Fibrin
4. Granulation tissue
5. New bone

Fig. 6.28 H&E X 40
5 Days (Col.4)

Zone of granulation tissue

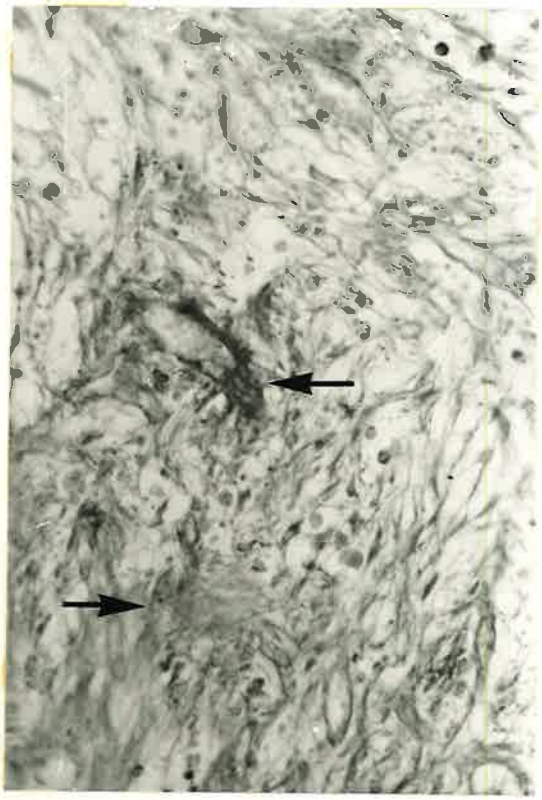


Fig. 6.29 PAS (no counter-stain) X 250
5 Days (Col.6)

Perivascular and interstitial PAS +ve material (arrows)

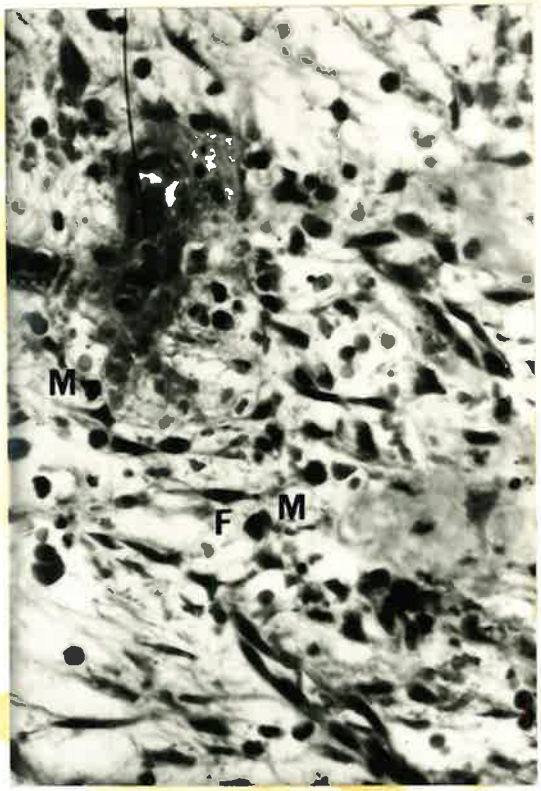


Fig. 6.30 H&E X 400
5 Days (Col.6)

Mitotic figures (M) close to capillaries
Fibroblasts (F)

Fig. 6.31 Silver X 100
5 Days (Col.4)

Argyrophilic fibres in granulation tissue

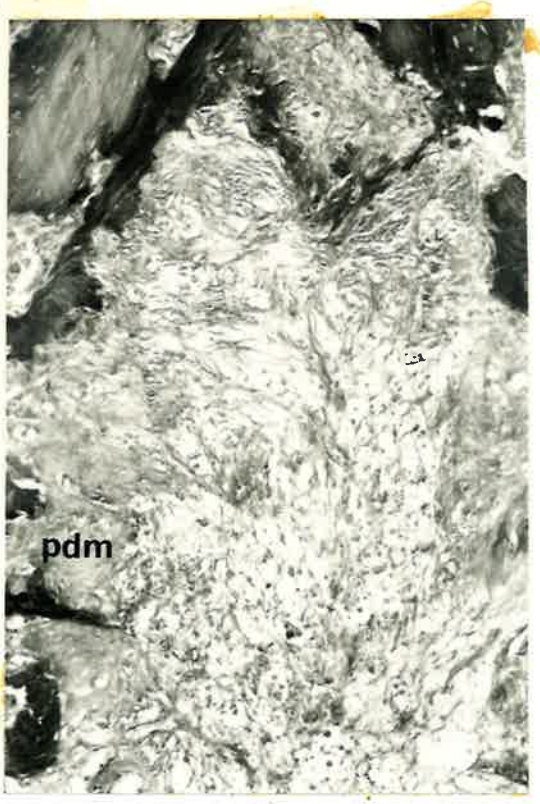


Fig. 6.32 VG X 100
5 Days (Col.6)

Collagen fibres in granulation tissue. Remnants of periodontal fibres (Pdm) has lost its fibrillar structure

Fig. 6.33 H&E X 25 5 Days (Col.3)

Gross resorption and inflammation in socket. Extensive periosteal bone formation (PB). Note inflammation (I) in extra-periosteal tissues buccally

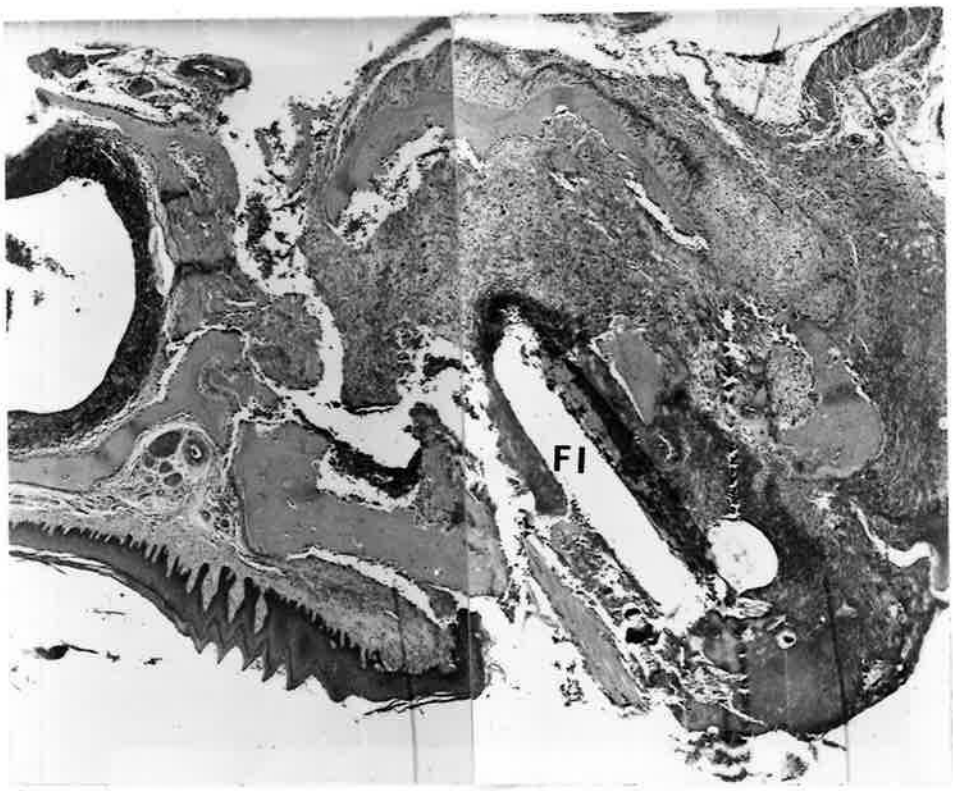


Fig. 6.34 H&E X 25 5 Days (Col.5)

Food impaction (FI) in socket and gross resorption of socket walls.
No new bone formation in socket, where there is intense inflammation.
Again, buccal tissues are inflamed.

Fig. 6.35 H&E X 100 5 Days (Col.3)

Intense osteoclastic
action in socket (Arrows)

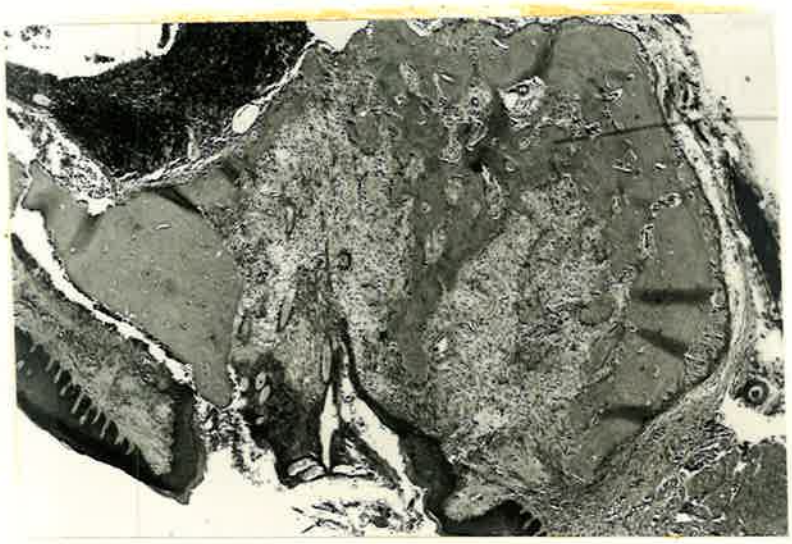


Fig. 6.36 H&E X 25 5 Days (Col.4)

Advanced state of healing.
Wound almost epithelialized.
Little inflammation in buccal
tissues.

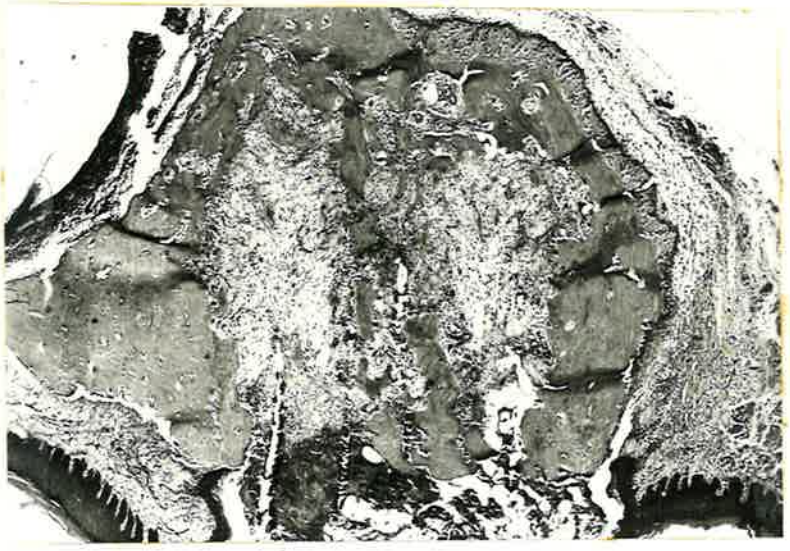


Fig. 6.37 H&E X 25 5 days (Col.6)

Less advanced healing in
socket compared with Fig. 6.36
Inflammation in buccal tissues

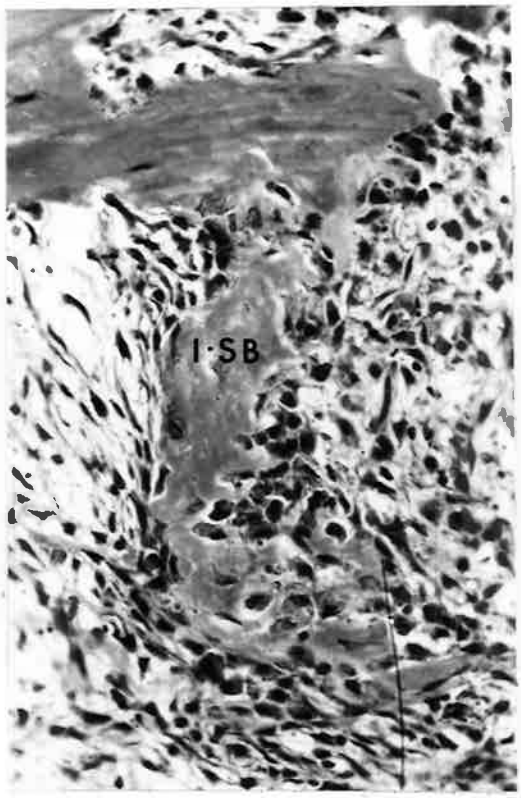


Fig. 6.38 H&E X 250
5 Days (Col.4)
New bone in socket (I-S B)

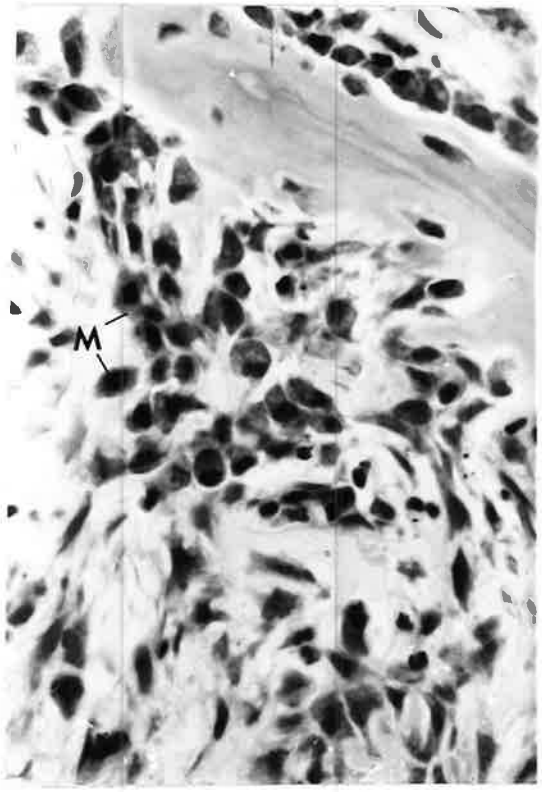


Fig. 6.39 H&E X 400
5 Days (Col.4)
Mitotic figures (M) in osteoblasts

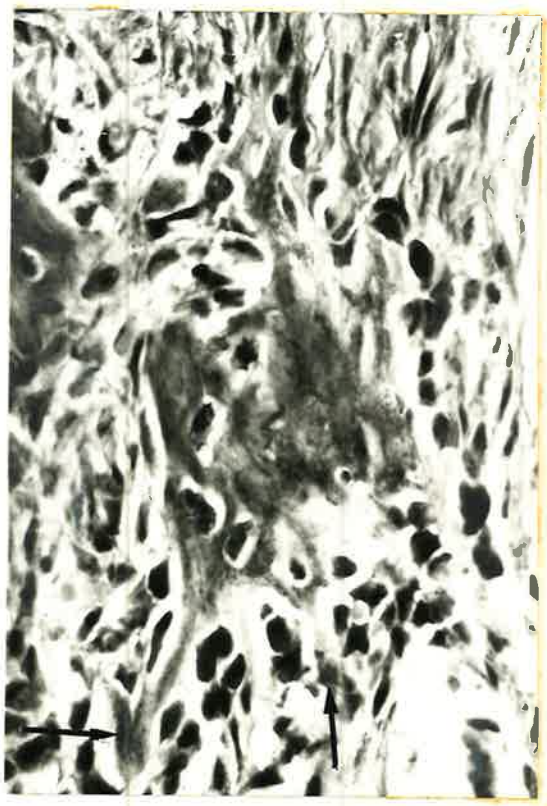


Fig. 6.40 PAS X 400
5 Days (Col.1)

PAS +ve bone matrix and intercellular material (arrows)

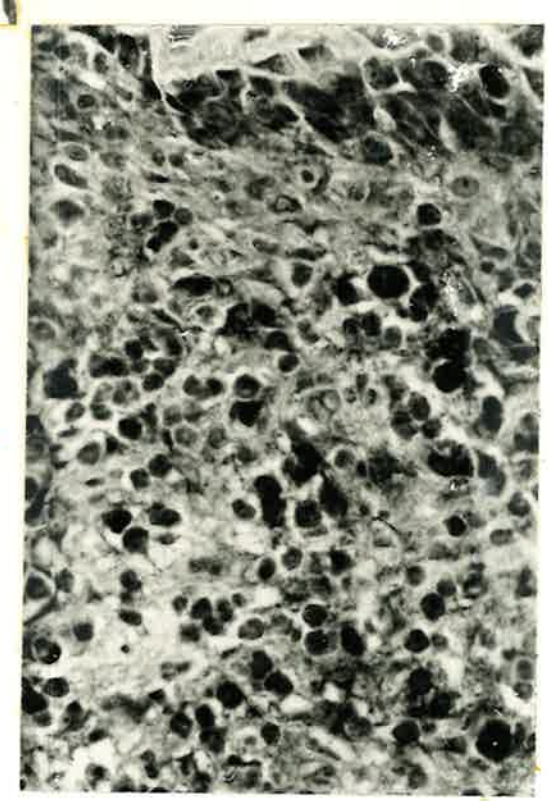


Fig. 6.41 Hale X 400
5 Days (Col.2)

Faintly staining acid mucopolysaccharide in interstitial areas amongst osteoblasts

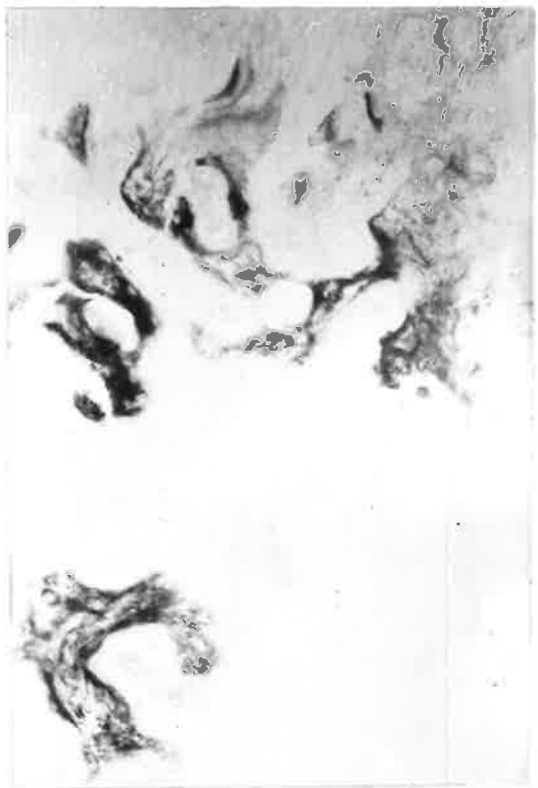


Fig. 6.42 CFP X 100
5 Days (T/CFP.3)

Chlorazol fast pink staining of bone matrix

Compare with Fig. 6.43

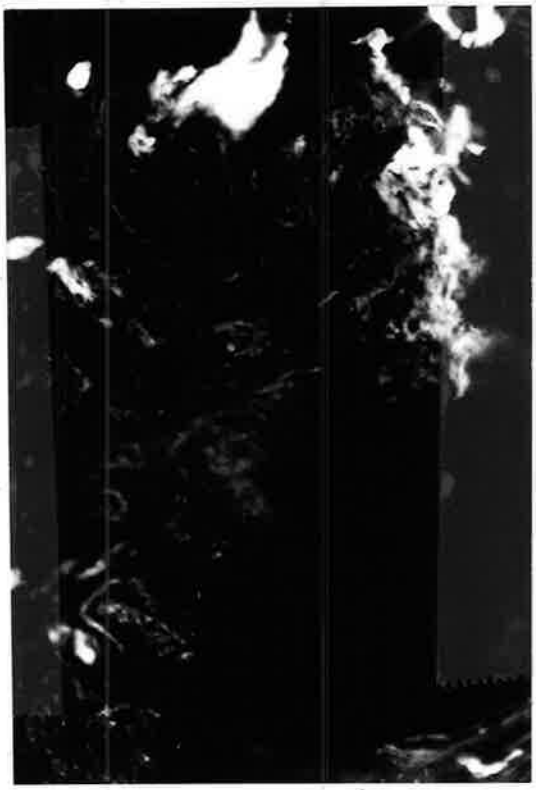


Fig. 6.43 Tetracycline X 100
5 Days (T/CFP.3)

Tetracycline fluorescence demonstrating mineralised portion of new bone in socket.

Compare with Fig. 6.42

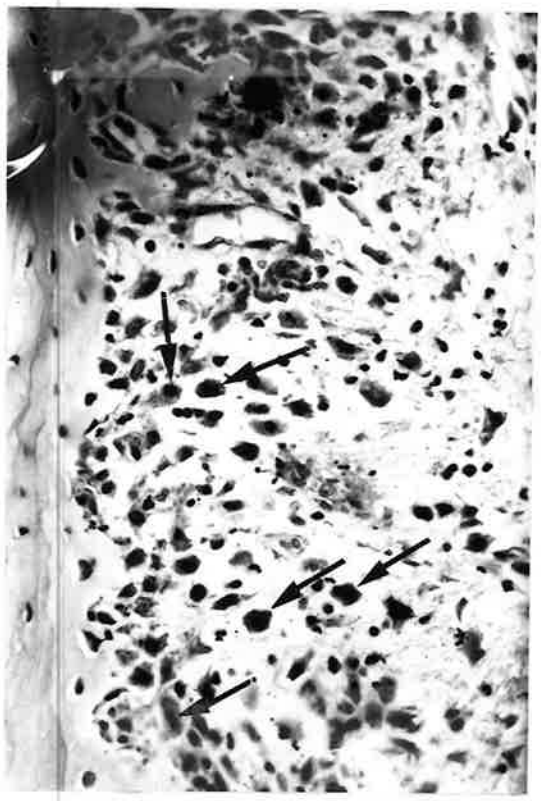


Fig. 6.44 H&E X 250 5 Days (Col.3)

Mitotic figures in the periosteal cells (arrows)

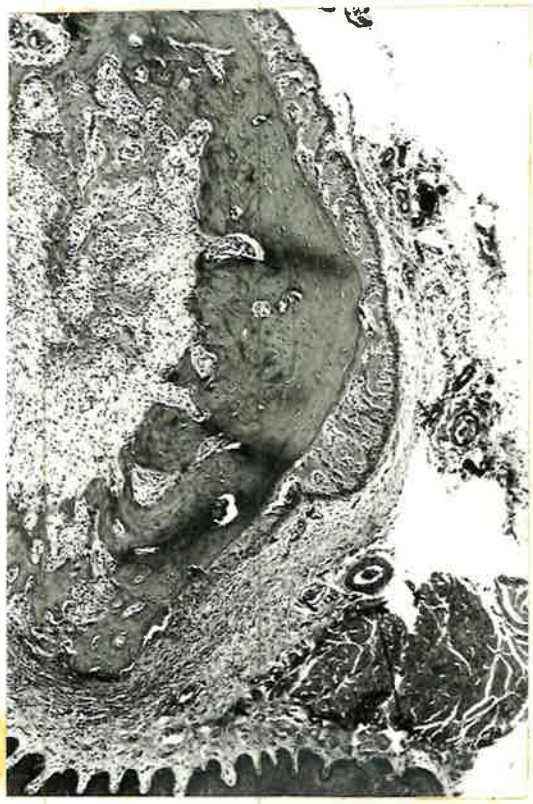


Fig. 6.45 H&E X 40
5 Days (Col.4)

Trabecular periosteal bone laid down at right angles to cortical bone

Fig. 6.46 H&E X 100
5 Days (Col.3)

Cortical bone (C).
Periosteal bone (PB).
Note edematous zone buccal to active periosteum.
Acid mucopolysaccharide accumulated in this zone (E), see Fig. 6.46

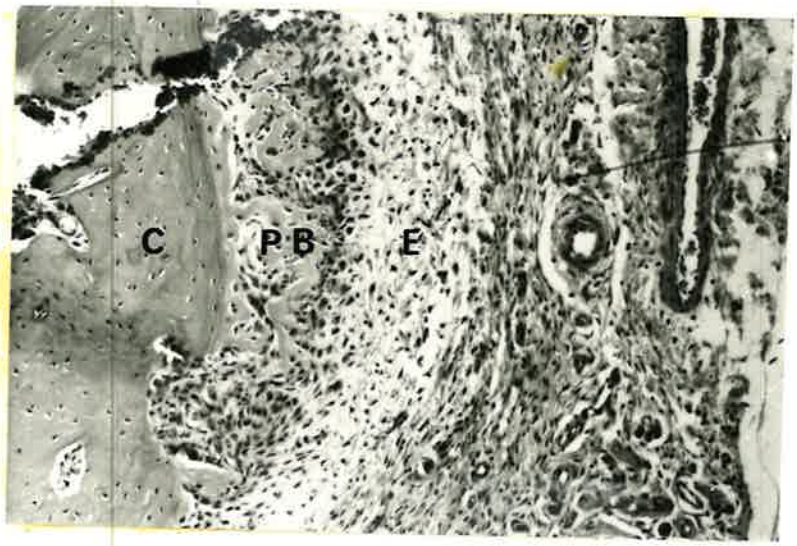
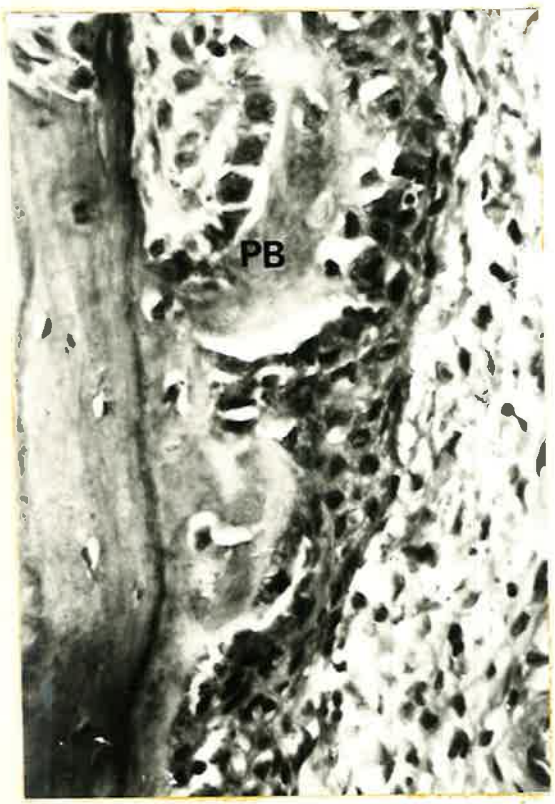


Fig. 6.47 PAS X 250
5 Days (Col.4)

Accumulation of PAS +ve material in interstitial areas between osteoblasts.
Periosteal bone (PB)



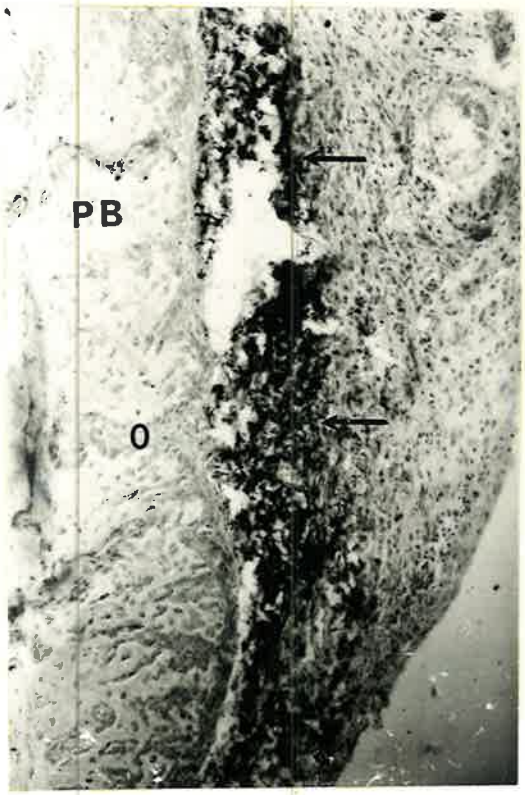


Fig. 6.48 Hale (no counter-stain) X 100

Acid mucopolysaccharide accumulation in edematous zone (arrows).
Periosteal bone (PB) not not stained.
Osteoblasts (O) faintly stained

Fig. 6.49 CFP X 100
5 Days (T/CFP.4)

Chlorazol fast pink staining of new bone matrix of periosteal bone

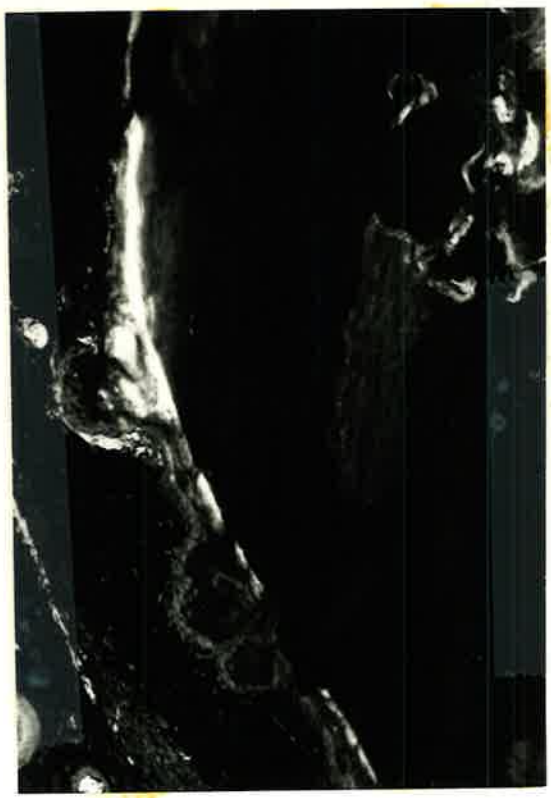


Fig. 6.50 Tetracycline X 100
5 Days (T/CFP.4)

Tetracycline fluorescence demonstrating mineralized portion of periosteal bone. Same location as Fig. 6.49

Fig. 6.51 Silver X 250 5 Days (Col.4)

Argyrophilic fibres in periosteal bone and periosteum. Cortical bone (CB).

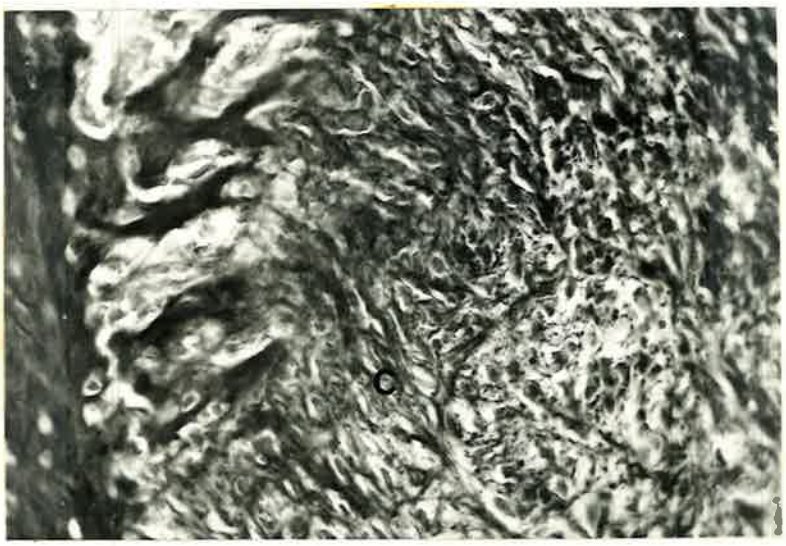


Fig. 6.52 VG X 250 5 Days (Col.4)

Collagen fibres (C) in close association with periosteal bone

7 days to 10 days

By the seventh post-operative day, the wound was epithelialized in sockets where healing had not been complicated by food impaction, or by sequestration of bone and root fragments. The inflammation had subsided and the socket had been filled entirely with granulation tissue (Figs. 6.53).

The granulation tissue in the socket still demonstrated the presence of argyrophilic fibres, but these were less distinct than in the 5-day specimens (Fig. 6.54). The intercellular fibres which were also more abundant now gave a more intense staining with Van Gieson's stain (Fig. 6.55). These fibres now gave a mild reaction with PAS, and a weak and diffused reaction with Hale's stain.

New trabecular bone had proliferated from the fundus and side of the sockets and had occupied the apical third of the alveolus (Fig. 6.53). Fig. 6.56 and 6.57 demonstrates the tetracycline stained new bone in the socket.

In specimens where healing had been uncomplicated, the amount of periosteal bone formed was minimal (Figs. 6.53, 6.58) and there was very little extra-periosteal inflammation (Fig. 6.59). The osteoblasts had returned to a less active state, very few mitotic figures were seen and the accumulation of PAS positive

material between these cells had decreased (Fig. 6.60). Hale's stain now gave a less intensive reaction (Fig. 6.61) in the periosteal tissues.

Fig. 6.62 shows a specimen in which healing was complicated by the sequestration of dead bone and food impaction. The socket content showed an intense inflammatory response. No new bone had formed in the socket. On the other hand, subperiosteal bone formation had been excessive and inflammation in extra-periosteal tissues was a noticeable feature. The continued accumulation of acid mucopolysaccharides in the periosteum is shown in Fig. 6.63.

Fig. 6.64 shows food impaction and associated inflammation in the palatal socket of a 7-day specimen. No new bone had been formed in the socket. However, bone had formed subperiosteally at the junction of the nasal and alveolar process of the maxilla where a nutrient canal opened on to the periosteal surface from the socket.

In two specimens, new bone formation on the endosteal surfaces of adjacent bone marrow spaces was also seen (Figs. 6.65, 6.66).

Specimen 10-day/col.4 (Figs. 6.67, 6.68) showed sequestration of bone and root fragments from the buccal socket. There was also

persistence of inflammation and gross periosteal bone formation and associated inflammation in extra-periosteal tissues. Compared with the above, Specimen 10 Days/col.6 showed the retention of a root fragment which had a vital pulp, which was not being sequestrated. In this specimen, there was little inflammation in the socket and extra-periosteal tissues, and the formation of periosteal bone had been minimal (Fig. 6.69).

Fig. 6.53 H&E X 25 7 Days (Col.2)

Advanced healing.
New bone in apical
third of socket (I-S B)
Minimal periosteal
bone (PB) formation

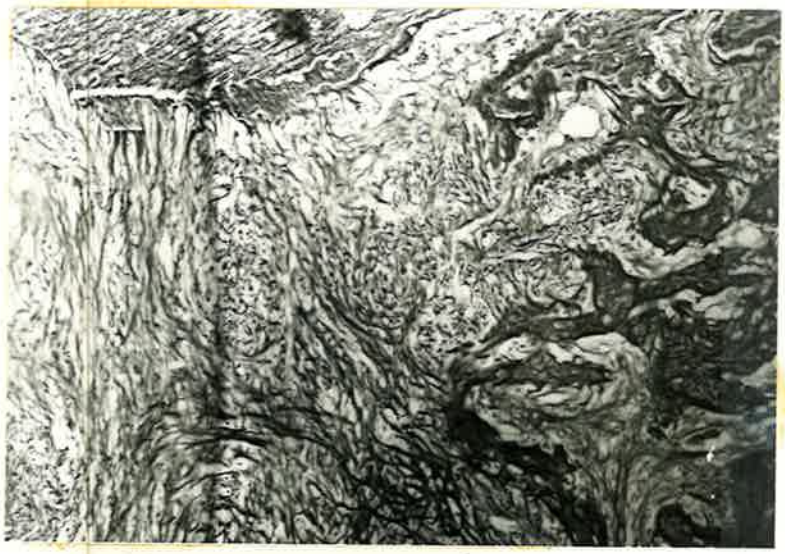


Fig. 6.54 Silver X 100 7 Days (Col.2)

Argyrophilic fibres in
granulation tissue

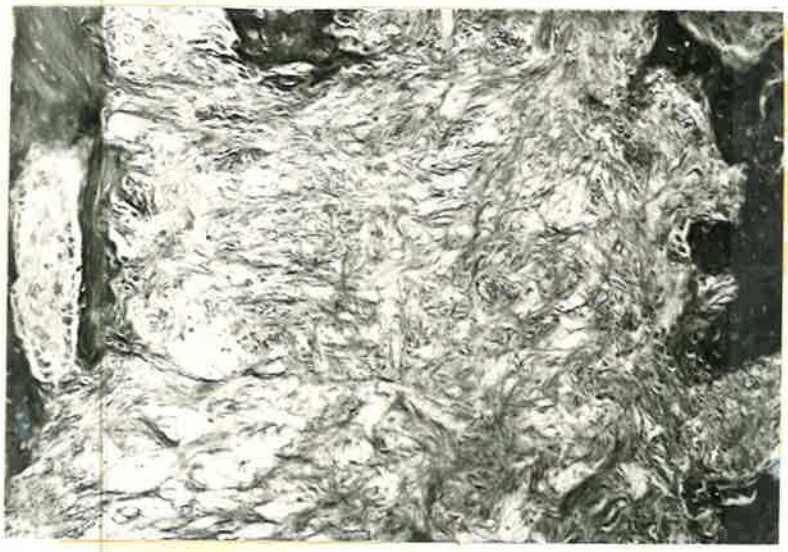


Fig. 6.55 VG X 100 7 Days (Col.1)

Collagen fibres in
granulation tissue
and on bone surface

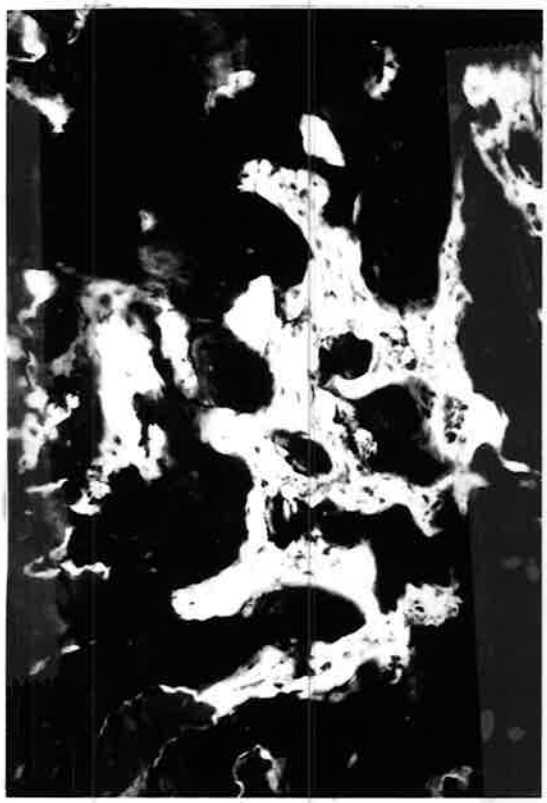


Fig. 6.56 Tetracycline X 100
7 Days (T/CFP.2)

Bone filling in from side and base of socket as demonstrated by tetracycline labelling

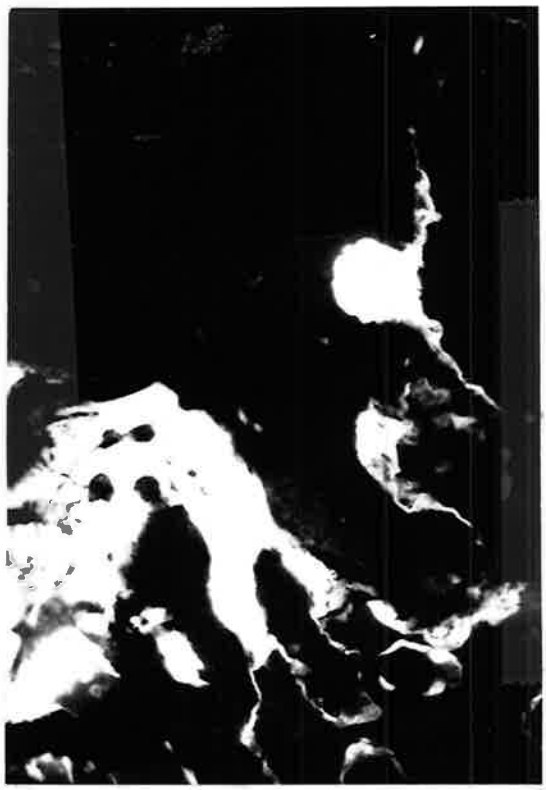


Fig. 6.57 Tetracycline X 100
7 Days (T/CFP.1)

Bone in side and base of socket as demonstrated by tetracycline labelling.

Fig. 6.58 Tetracycline
X 100
7 Days (T/CFP.1)

Small amount of periosteal bone formed (PB). Note auto-fluorescen in soft tissues (arrows)

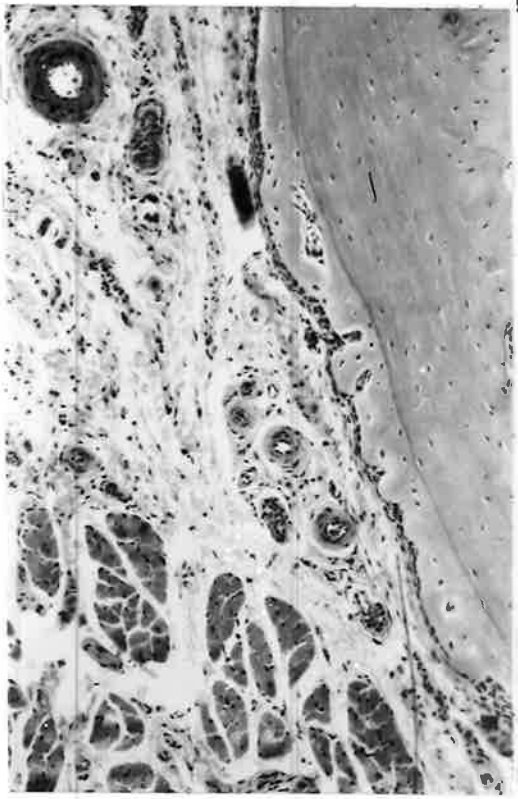


Fig. 6.59 H&E X 100
7 Days (Col.1)

Minimal periosteal bone formation and no inflammation in extra-periosteal tissues buccally.

Fig. 6.60 PAS X 250
7 Days (Col.2)

Much less PAS +ve material between osteoblasts than in Fig. 6.47.
Osteoblasts in less active state

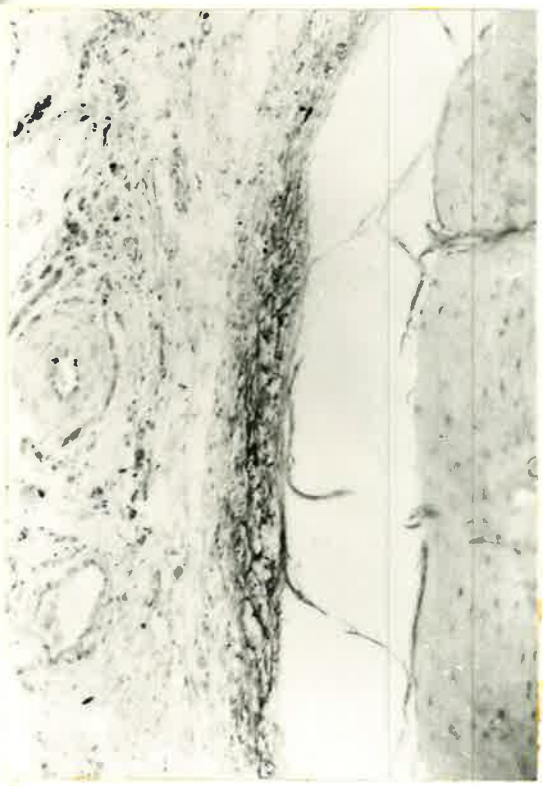


Fig. 6.61 Hale X 100
7 Days (Col.2)

Decrease in amount of acid mucopolysaccharide in periosteal tissues.
Compare with Fig. 6.48.

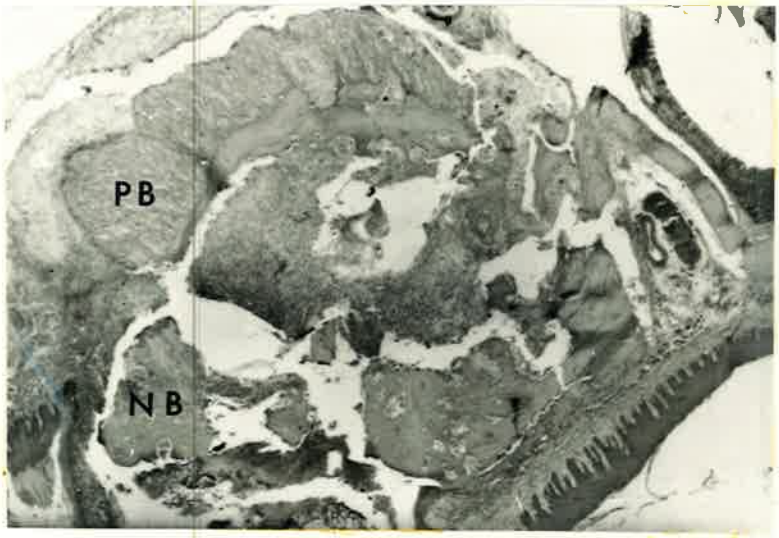


Fig. 6.62 H&E X 25 7 Days (Col.4)

Sequestration of necrotic bone (NB).
Granulation tissue in socket still intensely inflammed.
Gross periosteal bone formation (PB).

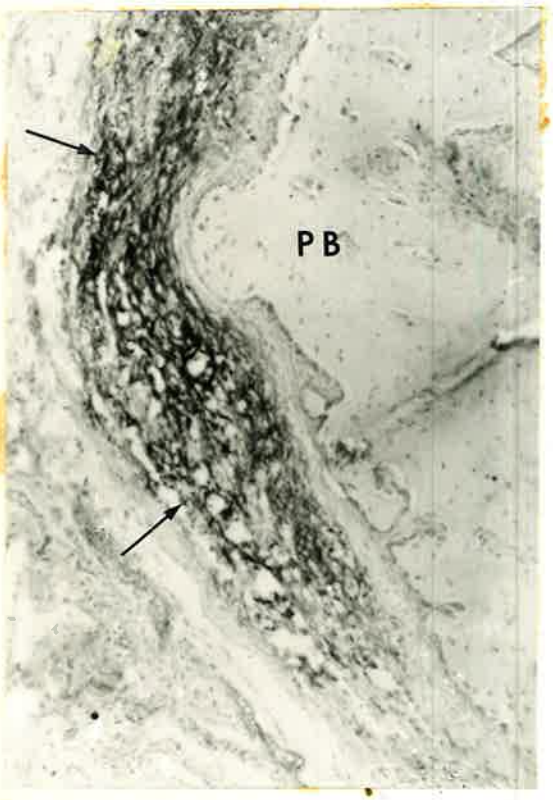


Fig. 6.63 Hale X 100
7 Days (Col.4)

Acid mucopolysaccharide (arrows) accumulation in periosteal tissues where new bone (PB) formation has been gross

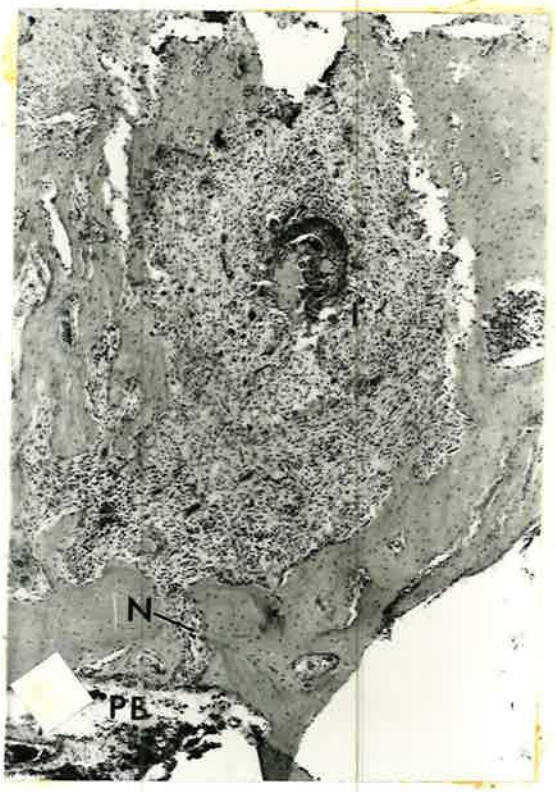


Fig. 6.64 H&E X 40
7 Days (Col.3)

Inflammation in socket (I). Nutrient canal (N) leading from socket to periosteal surface. Note periosteal bone (PB) formation at opening of nutrient canal

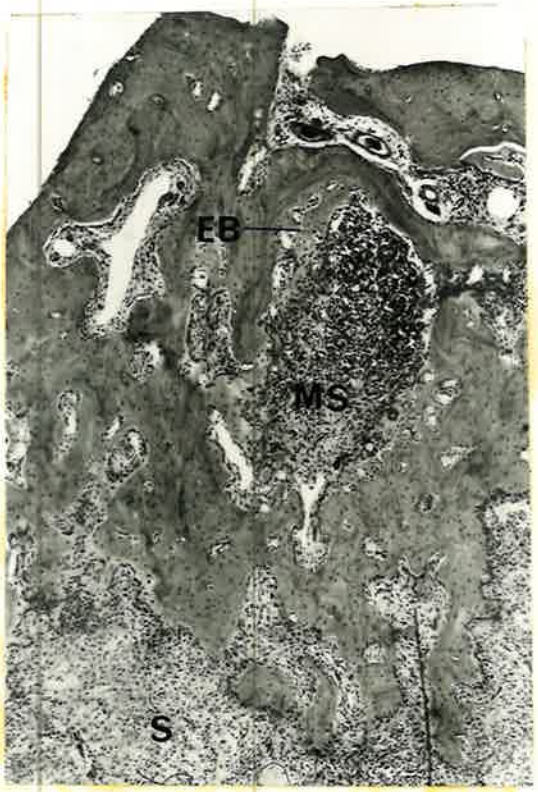
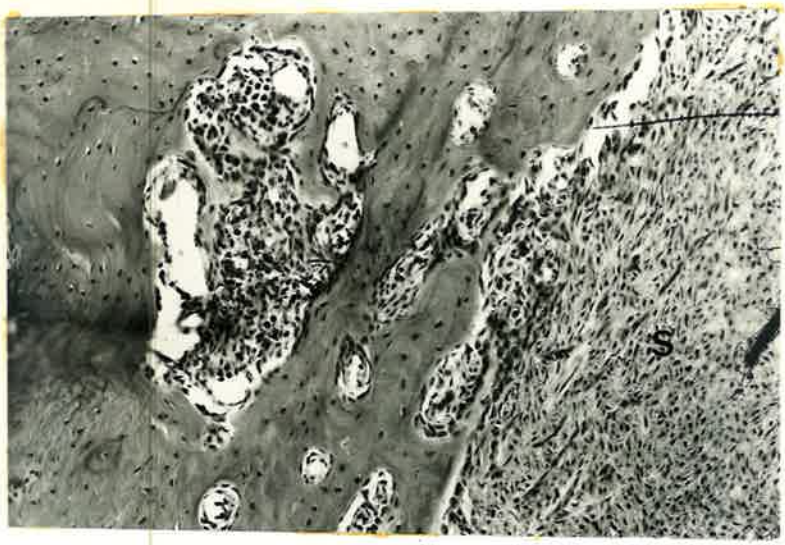


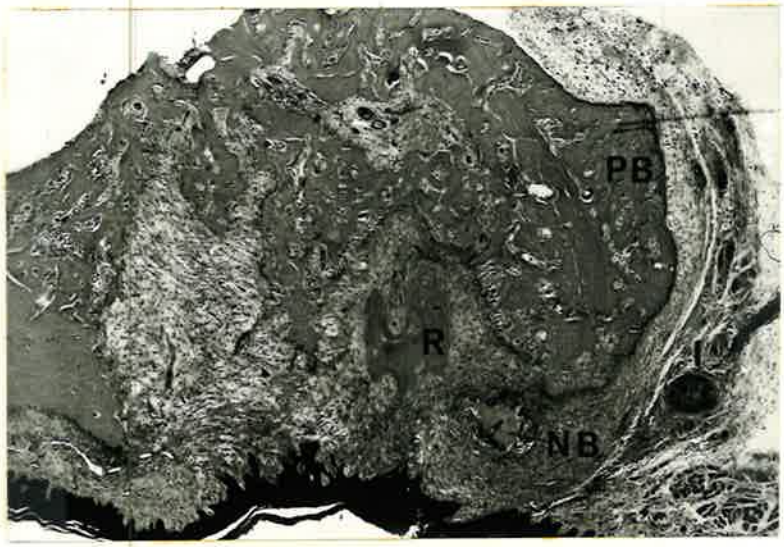
Fig. 6.65 H&E X 40
7 Days (Col.2)

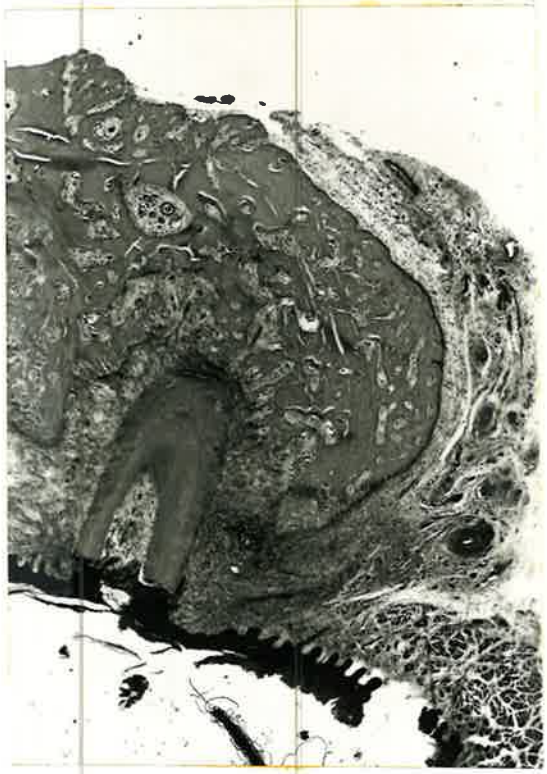
New bone (EB) formation on endosteal surface of adjacent marrow space (MS). Socket (S).

Fig. 6.66 H&E X 40
7 Days (Col.2)

Endosteal bone formation in adjacent marrow space. Socket (S)







Figs. 6.67 and 6.68

H&E X 25 10 Days (Col.4)
Serial sections

Sequestration of necrotic bone (NB) and root fragment (R).
Inflammation (I) in buccal tissues evident.
Extensive periosteal bone (PB) formation.

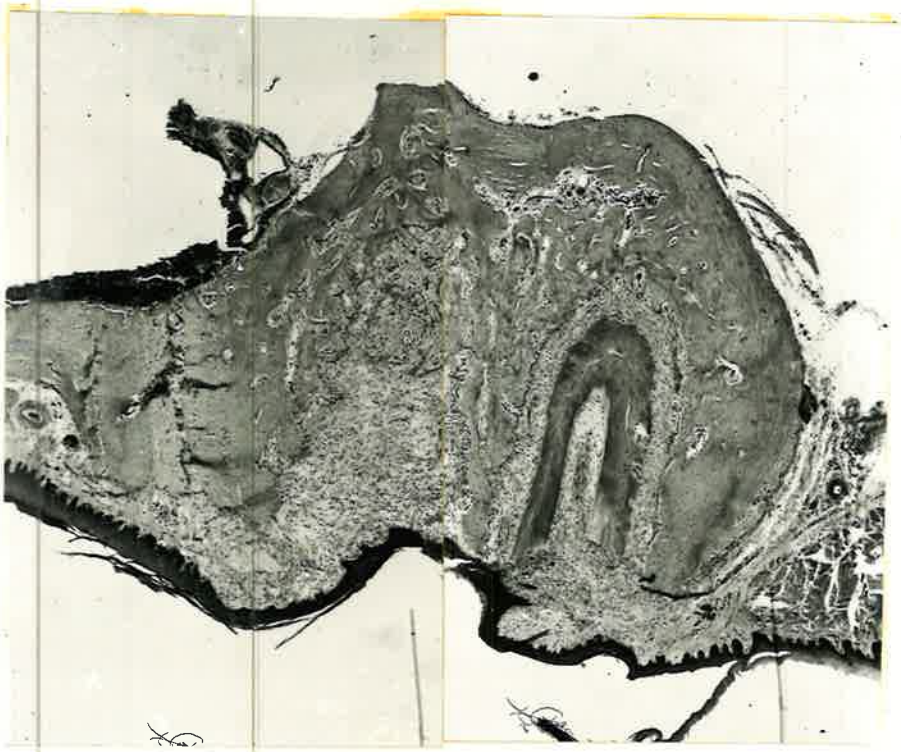


Fig. 6.69 H&E X 25 10 Days (Col.6)

Retention of a vital root
in buccal socket.
Note lack of inflammation and
minimal periosteal bone
formation

14 Days

The granulation tissue in the socket was by now more mature, and contained collagen fibres which showed orientation (Figs. 6.70, 6.71). Blood vessels were less evident and argyrophilic fibres were less distinct (Fig. 6.72). These fibres showed the same orientation as the collagen fibres. The intercellular and ground substances were now only weakly PAS positive and showed no staining for mucopolysaccharide with Hale's stain.

At this stage these uncomplicated healing sockets were filled to two-thirds by new bone (Figs. 6.73, 6.74, 6.77). In these specimens, the amount of periosteal bone formed was limited in quantity (Figs. 6.73, 6.74, 6.78) and mature in structure (Fig. 6.81). There was little inflammation in the extra-periosteal tissues. The osteoblasts had either reverted to a state of inactivity with small, spindle shaped nuclei (Fig. 6.80) similar to the cambium layer of cells on the control side (Fig. 6.79) or were a single layer of cuboidal cells with plump nuclei but no mitosis (Fig. 6.81).

In contrast, where there was food impaction, and persistence of intense inflammation, new bone had not been formed in the socket (Fig. 6.75) and extensive resorption of the alveolar

socket had taken place. Fig. 6.76 shows a specimen in which a large piece of necrotic bone is being sequestrated, but underneath which the inflammatory reaction is not severe, new bone in the socket had also been formed.

In these latter specimens, where there had been a complication in healing there was a large amount of trabecular periosteal bone formed (Figs. 6.75, 6.76). The extraperiosteal tissues were still inflamed, the osteoblasts were active and showed mitosis (Fig. 6.83) and the edematous zone outside this layer of cells still contained acid mucopolysaccharides as demonstrated by Hale's stain (Fig. 6.84).

Osteoclastic resorption in the socket was not seen except where there was persistence of inflammation. However, in well healed sockets resorption of the tip of the buccal cortical plate was noted in cases when this had not been covered by a layer of new periosteal bone (Fig. 6.85).

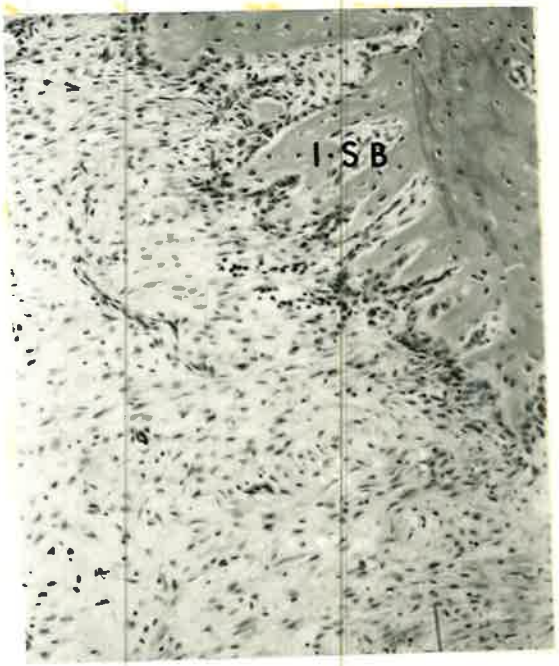


Fig. 6.70 H&E X 100
2 wks. (Col.2)

New bone in socket (I-S B).
Granulation tissue more
mature compared with
Fig. 6.71

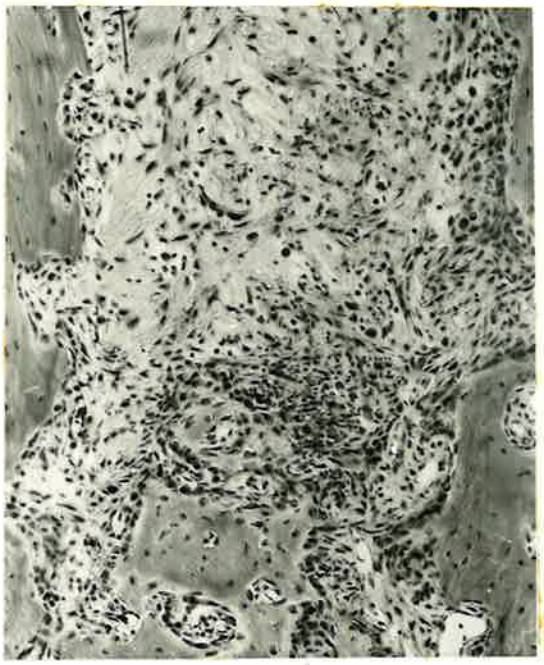


Fig. 6.71 H&E X 100
7 Days (Col.6)

Young granulation tissue
in socket with immature
collagen fibres

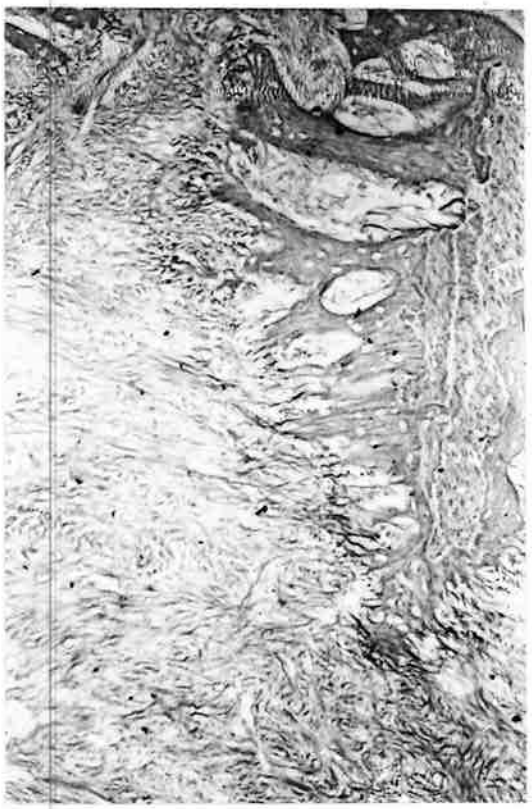


Fig. 6.72 Silver X 100
2 Wks. (Col.2)

Argyrophilia in granulation tissue much less pronounced than earlier stages

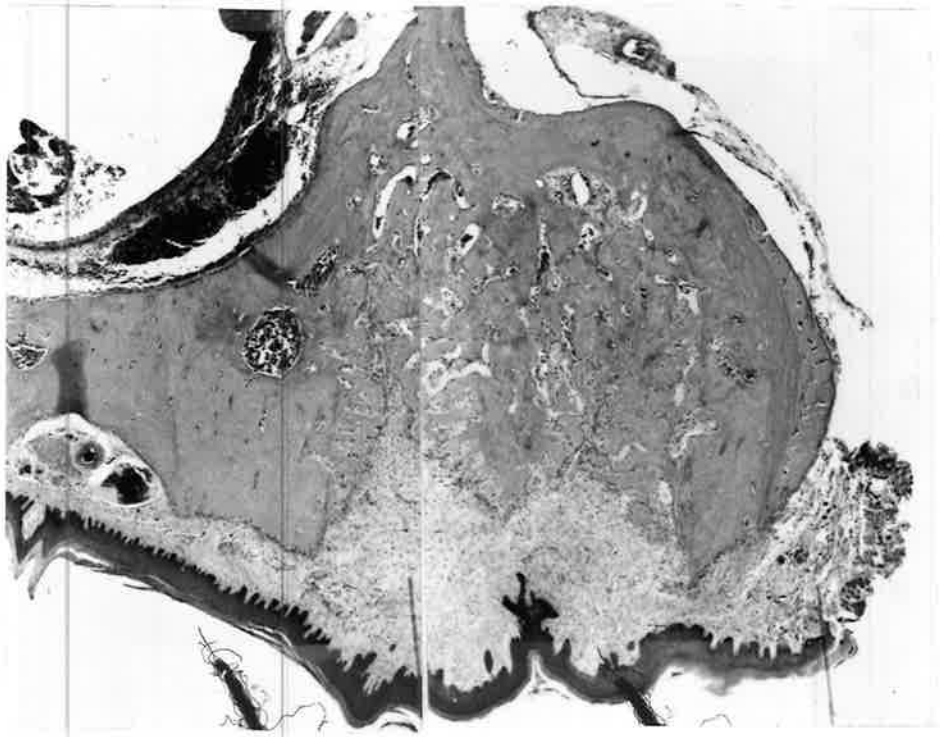


Fig. 6.73 H&E X 25 2 Wks.(Col.2)

Socket filled to two thirds
with new bone.
Minimal periosteal bone formation.

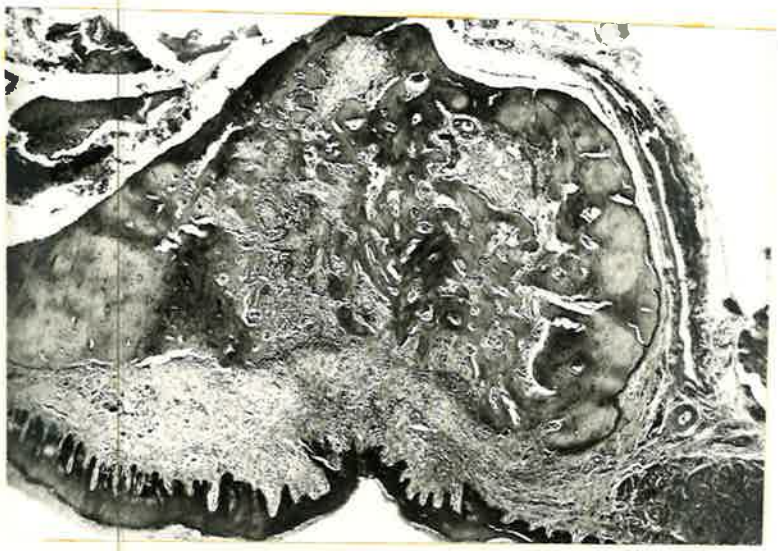


Fig. 6.74 H&E X 25 2 Wks.(Col.1)

Socket two-thirds filled with bone. Minimal periosteal bone formation.

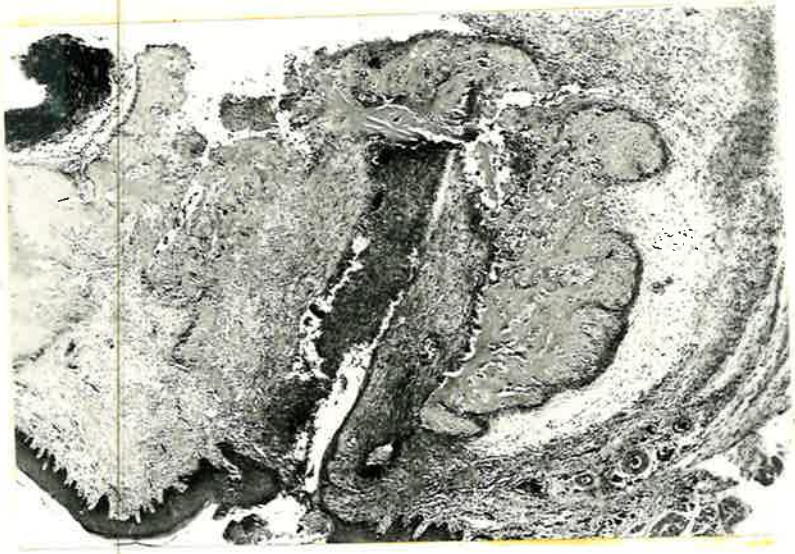


Fig. 6.75 H&E 2 Wks.(Col.4)

Food impaction in socket with inflammation and gross resorption. Extensive periosteal bone formation

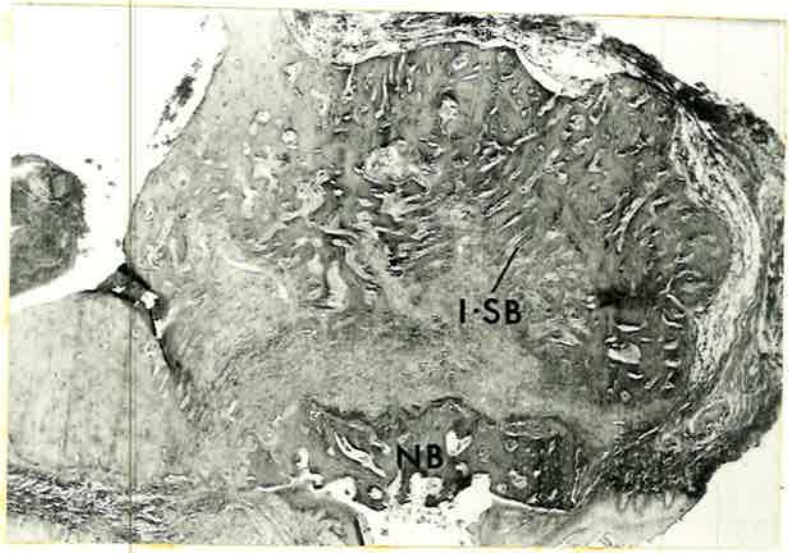


Fig. 6.76 H&E X 25 2 Wks.(Col.5)

Sequestration of necrotic bone (NB). Relatively mild inflammation in socket. Intra-socket bone (I-S B)

Fig. 6.77 Tetracycline X 40 2 Wks.(T/CF).

New bone formation in palatal socket demonstrated by tetracycline fluorescence.

Fig. 6.78 Tetracycline
X 40
2 Wks.(T/CFP.4)

Minimal amount of new
bone formed subperiosteally

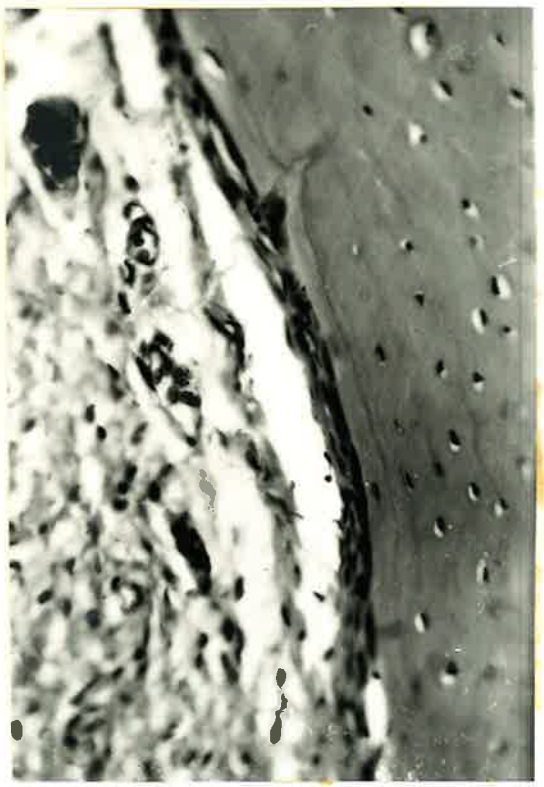


Fig. 6.79 H&E X 250
2 Wks.(Col.7)

Control side pericsteum

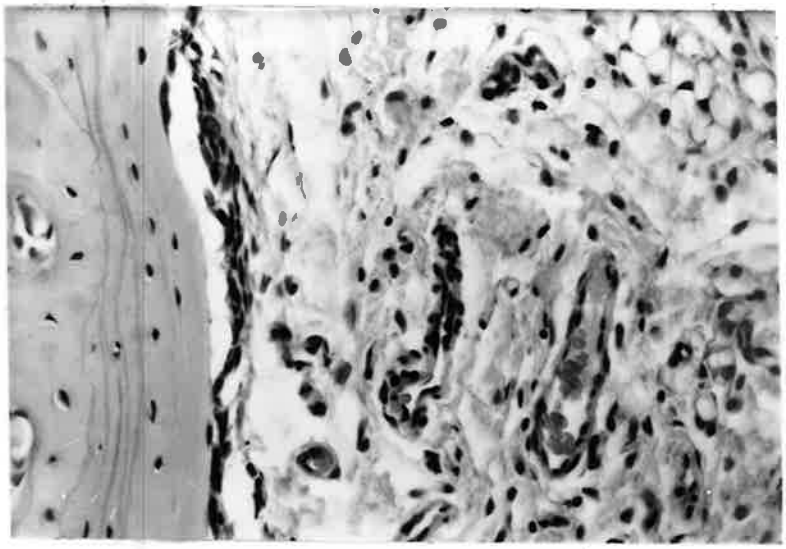


Fig. 6.80 H&E X 250 2 Wks.(Col.7)
Periosteum now inactive

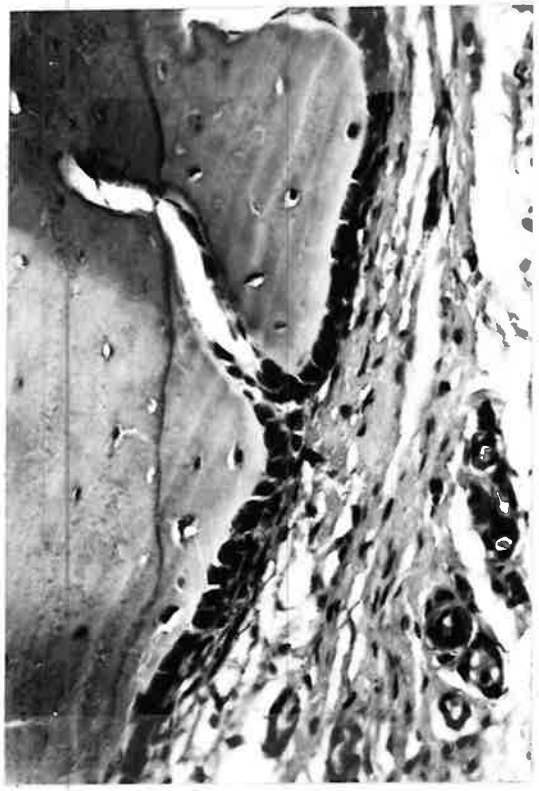


Fig. 6.81 H&E.X 250
2 Wks.(Col.1)

Single layer of
cuboidal osteoblasts

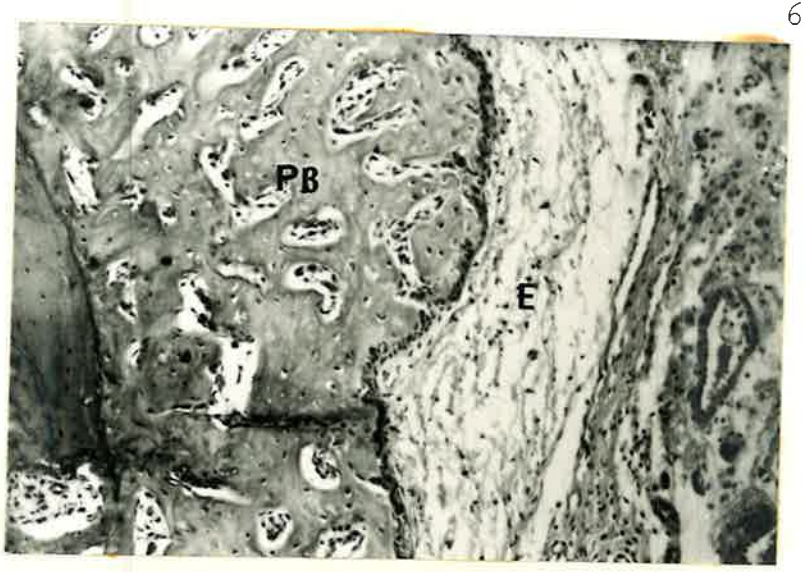


Fig. 6.82 H&E X 100 2 Wks.(Col.5)

Gross periosteal bone (PB). Note edematous zone (E), where acid mucopolysaccharide accumulates (see Fig. 6.84)

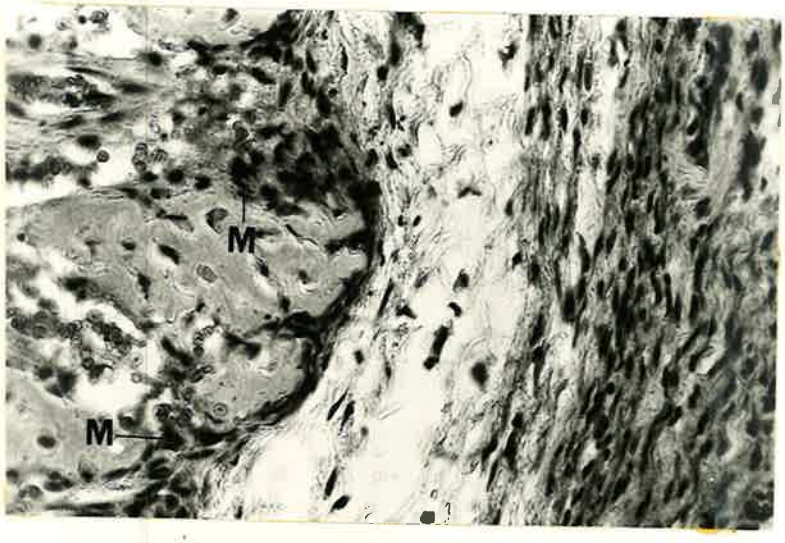


Fig. 6.83. H&E X 250 2 Wks.(Col.4)

Gross amount of periosteal bone. Mitotic figures (M) in still active periosteum

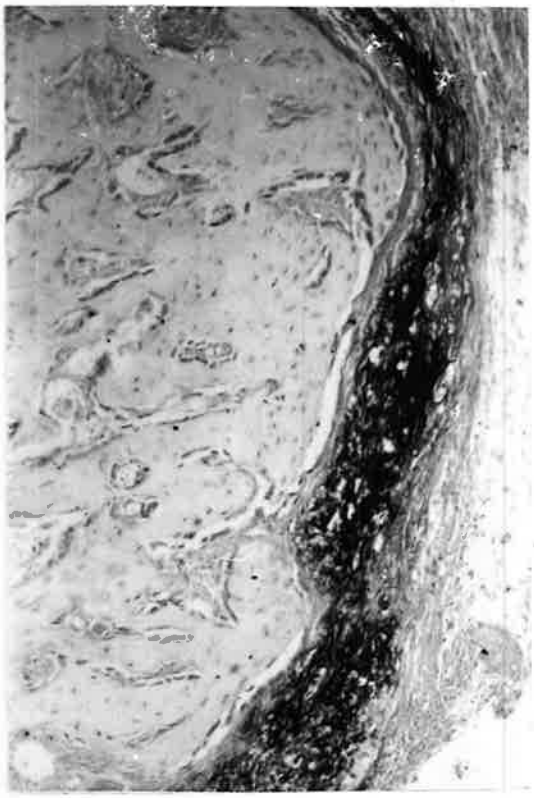


Fig. 6.84 Hale X 100
2 Wks.(Col.5)

A particularly heavy accumulation of acid mucopolysaccharide in periosteal tissues,where bone formation continues.

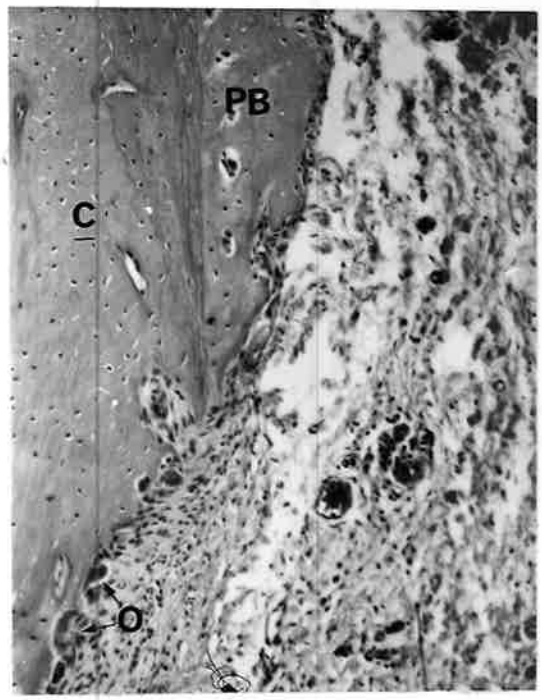


Fig. 6.85 H&E X 100
2 Wks.(Col.2)

Buccal alveolar crest region.
Cortical bone (C)
Periosteal bone (PB)
Note osteoclastic resorption (O) of cortical bone where it had not been covered by periosteal bone

3 Weeks to 4 Weeks

The granulation tissue continued to undergo maturation and was progressively replaced by new bone filling in from the sides of the socket walls and from the fundus (Fig. 6.86).

By the end of the third week, the remaining granulation tissue consisted mainly of mature collagen fibres which were parallel to the bone surface (Fig. 6.88, 6.89). In specimens which showed advanced healing within the socket, there was no sign of inflammation either in the remaining granulation tissue, or in the extra-periosteal tissue buccally. In these specimens there was little bone formed subperiosteally (Fig.6.86, 6.91, 6.92) and its formation had been limited to the first three weeks of healing. This was demonstrated by the fact that periosteal bone was confined by the two labelling fluorescent bands, one produced by injection on the 7-8th days and the other by injection on the 19th-20th days (Figs. 6.91, 6.92).

In specimens where sequestration of bone and root fragments occurred, there was a decline in the inflammatory reaction as the process was completed (Figs. 6.87, 6.93, 6.95, 6.97). These specimens showed varying amounts of bone formed within the socket (Figs. 6.93, 6.94, 6.95, 6.96, 6.97, 6.98) and an accompanying extensive periosteal bone formation.

Remodelling of bone had resulted in resorption of the tips of the buccal alveolar crests to produce a rounded appearance, blending with the new bone in the socket (Figs. 6.86, 6.87, 6.90). The bone laid down in the socket and that subperiosteally was now dense in structure (Figs. 6.86, 6.87, 6.99).

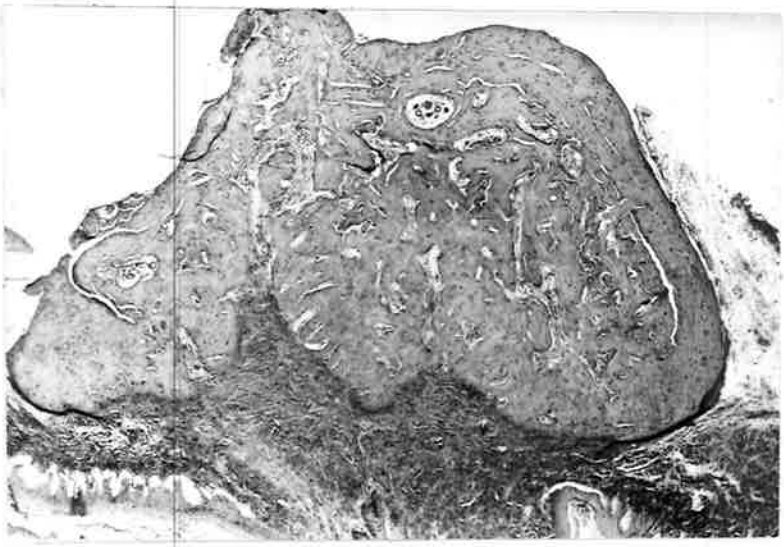


Fig. 6.86 H&E X 25 3 Wks.(Col.1)

Socket almost completely filled with new bone. Note lack of periosteal bone formation.

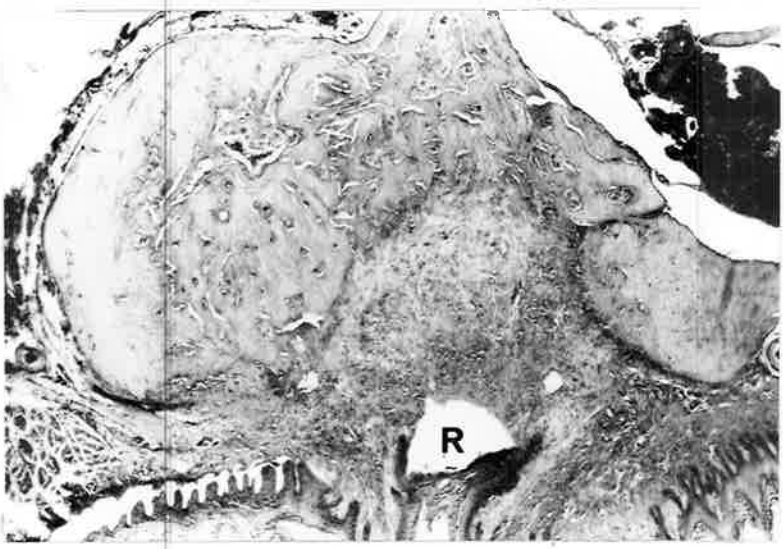


Fig. 6.87 VG X 25 Wks.(Col.1)

Socket still not filled in by new bone completely. Note space occupied by sequestrating root fragment (R).

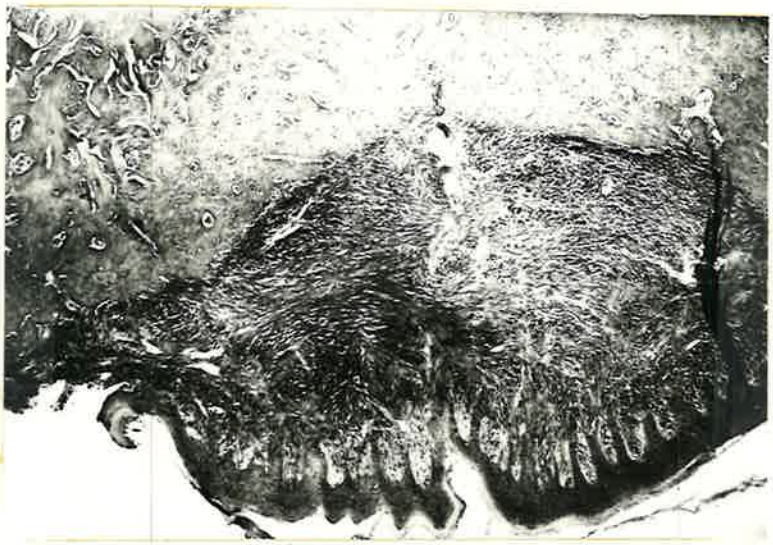


Fig. 6.88 VG X 40 3 Wks.(Col.1)

Showing residual granulation tissue.

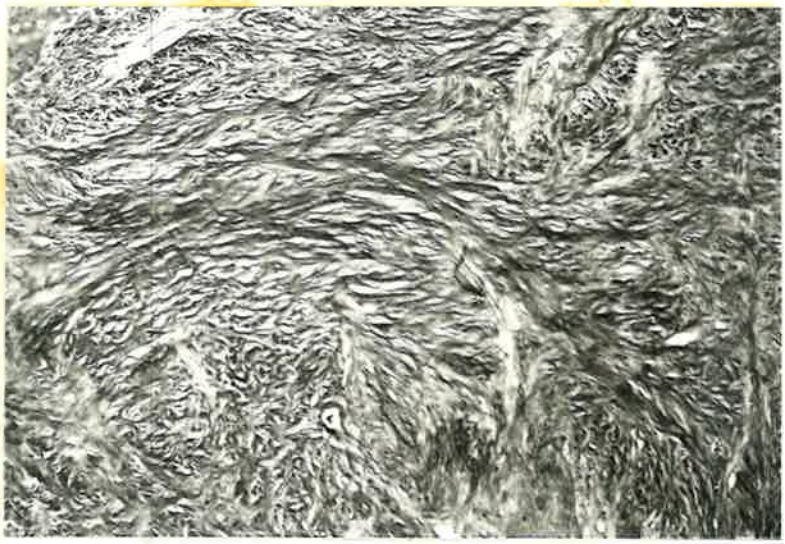


Fig. 6.89 VG X 100 3 Wks.(Col.1)

High power magnification of Fig. 6.89. Note direction of collagen fibres.

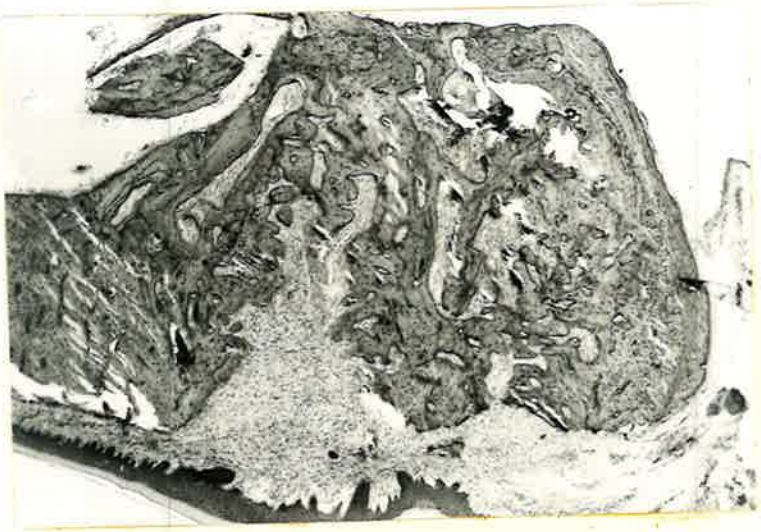


Fig. 6.90 H&E X 25 4 Wks.(T/CFP.1)

General view of socket region.
Serial section of Fig. 6.91.

Fig. 6.91 Tetracycline X 25
4 Wks.(T/CFP.1)

Tetracycline labelling showing areas of new bone formation.

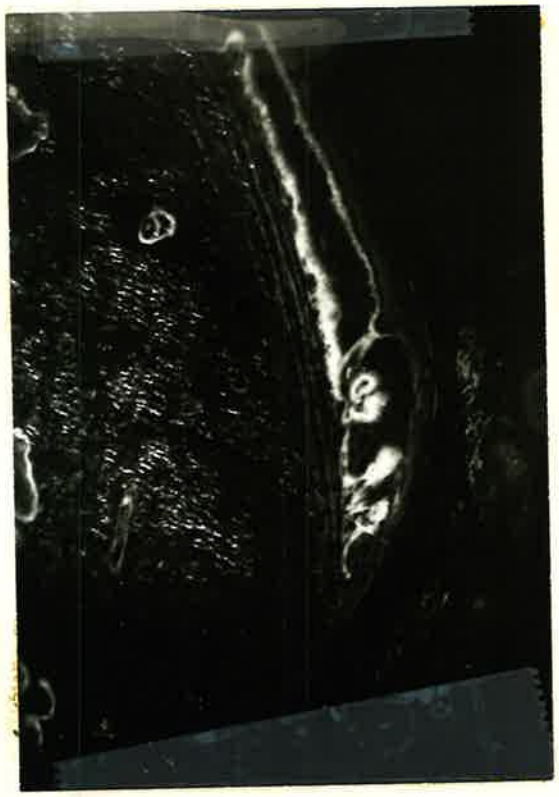


Fig. 6.92 Tetracycline X 40
4 Wks.(T/CFP.1)

Magnification of buccal alveolar crest area in Fig. 6.91
Limited amount of periosteal bone.

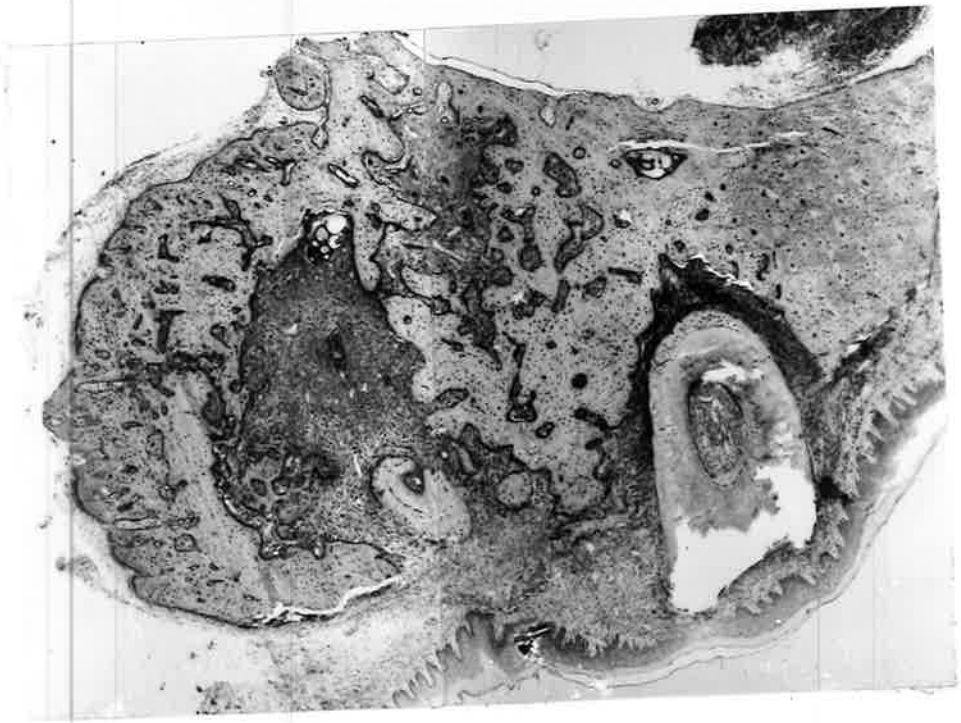


Fig. 6.93 Methylene blue X 25
4 Wks.(T/CFP. 3)

Sequestration of root fragments.
Serial section of Fig. 6.94

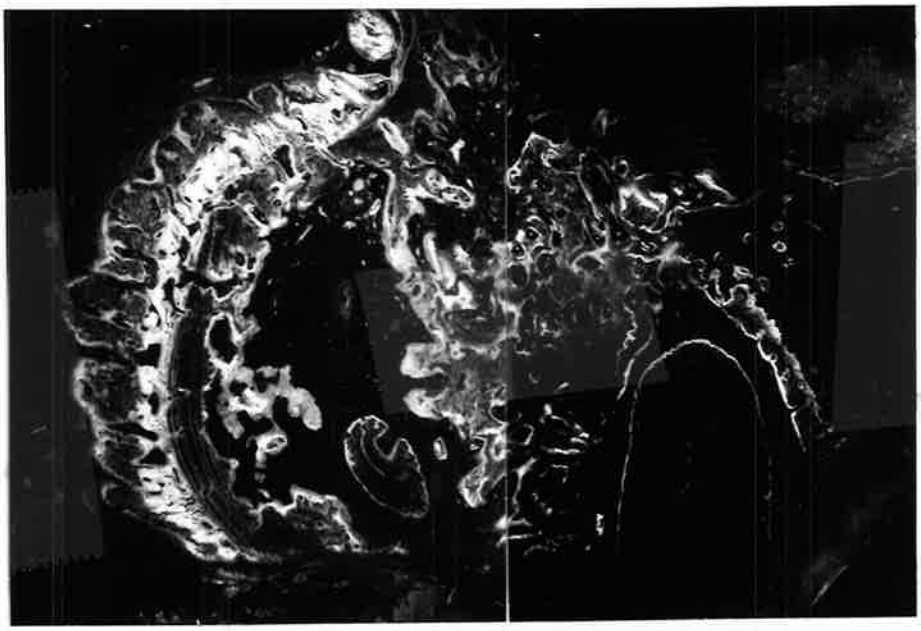


Fig. 6.94 Tetracycline X 40
4 Wks.(T/CFP.3)

Compared with Fig. 6.91, this specimen shows much more pronounced periosteal bone formation and much less intra-socket bone.

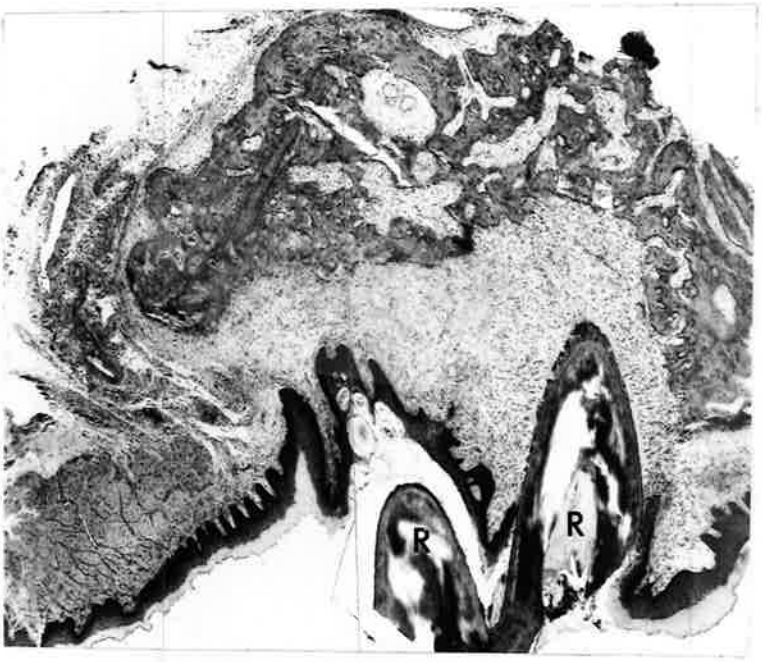


Fig. 6.95 H&E 4 Wks.(T/CFP.4)
Sequestration of root fragments (R)

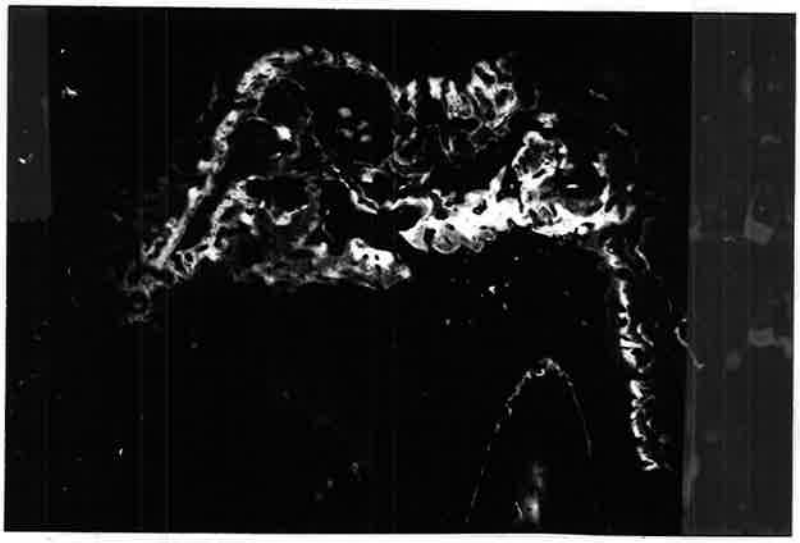


Fig. 6.96 Tetracycline X 25 4 Wks.(T/CFP.4)
Tetracycline fluorescence showing the amount of periosteal and intra-socket bone formed

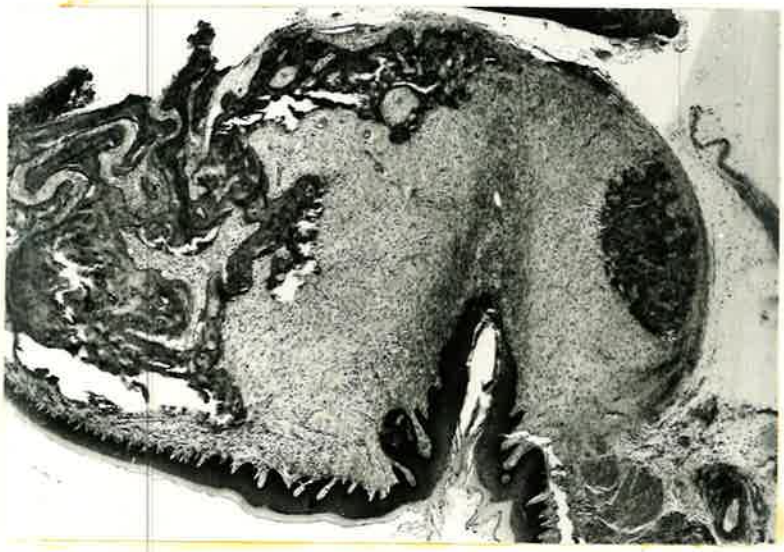


Fig. 6.97 H&E X. 25 4 Wks.(T/CFP.2)

Food impaction in socket, persistent inflammation and extensive resorption of socket.

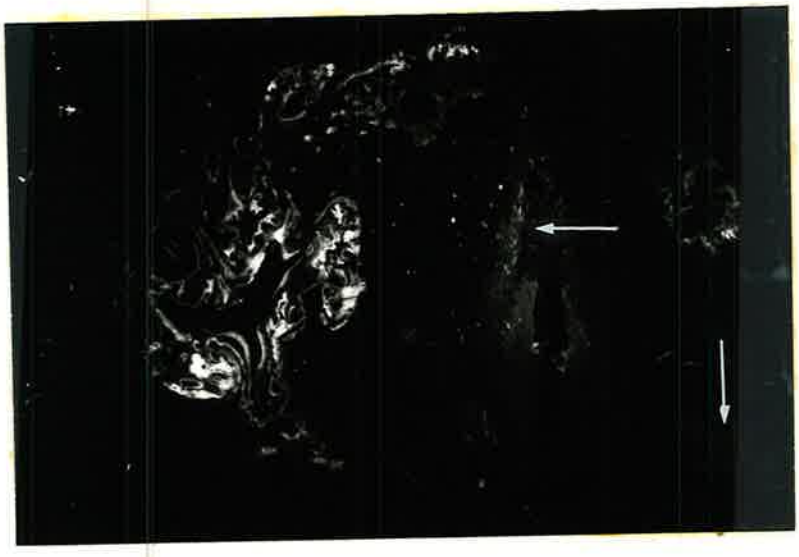


Fig. 6.98 Tetracycline X 25
4 Wks (T/CFP.2)

Tetracycline labelled new bone. Auto-fluorescence in soft tissues (arrows)

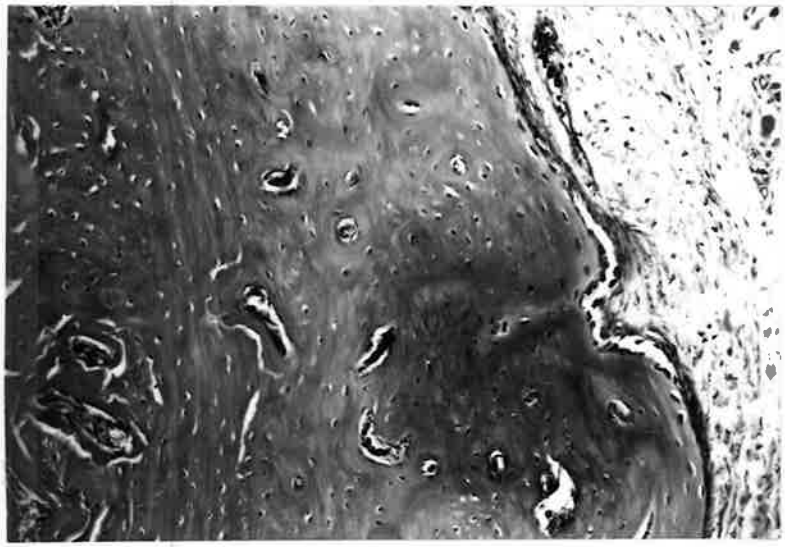


Fig. 6.99 4 Wks.(Col.4) H&E X 100

Dense periosteal bone and inactive periosteum

8 Weeks

This period was essentially one of maturation and remodelling of bone. Formation of bone both in the socket and subperiosteally had ceased. Remodelling had produced a rounded buccal alveolar crest (Figs. 6.100, 6.109). Figs. 6.100, 6.102, 6.104 show the general outline of the socket area at this period.

Tetracycline fluorescence (Figs. 6.101, 6.103, 6.105) demonstrates the amount of periosteal bone formation in three 8 weeks specimens. It can been seen that periosteal bone formation in these specimens had mainly been laid down during the first three post-operative weeks. Most of the periosteal bone was labelled by the first and second tetracycline injection administered on the 7th-8th and 19th-20th days.

The amount of bone between the second and third bands indicated by results of injection on the 19th-20th and 33rd-34th days) was relatively narrow in extent (Figs. 6.107, 6.108.) A comparison of the amount of periosteal bone laid down during this period on the experimental sides with that on the control side shows that there is little difference in the amount formed during this period (Figs. 6.106, 6.107, 6.108). This

indicates that bone formed in the experimental animal during the 4th and 5th post-operative week was therefore not in excess of that resulting from normal growth.

Fluorescence in the third labelling band was weak in intensity, and in some locations not present. This could have been due to either a lack of bone deposition during the injection period or a loss of labelled bone through remodelling.

Bone formed in the socket was now more mature in structure. Because of the irregular and complex manner in which bone was laid down in the socket, it was often not possible to trace labelled bands laid down during the various periods. Consequently, it was not possible to determine for the bone of any particular period which portion was being remodelled.

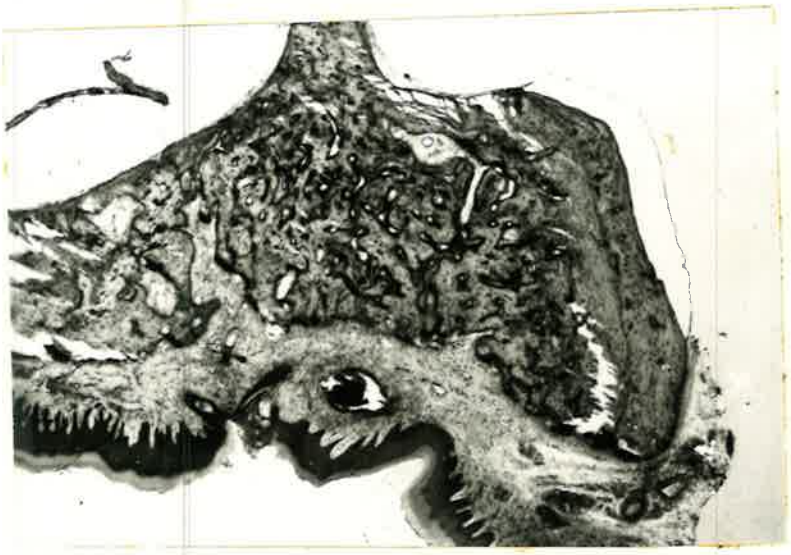


Fig. 6.100 H&E X 25 8 Wks.(T/CFP.2)

General view of alveolar region.
Serial section of Fig.6.101.

Fig. 6.101 Tetracycline X 25
8 Wks.(T/CFP.2)

Tetracycline labelling of intra-socket and periosteal bone.

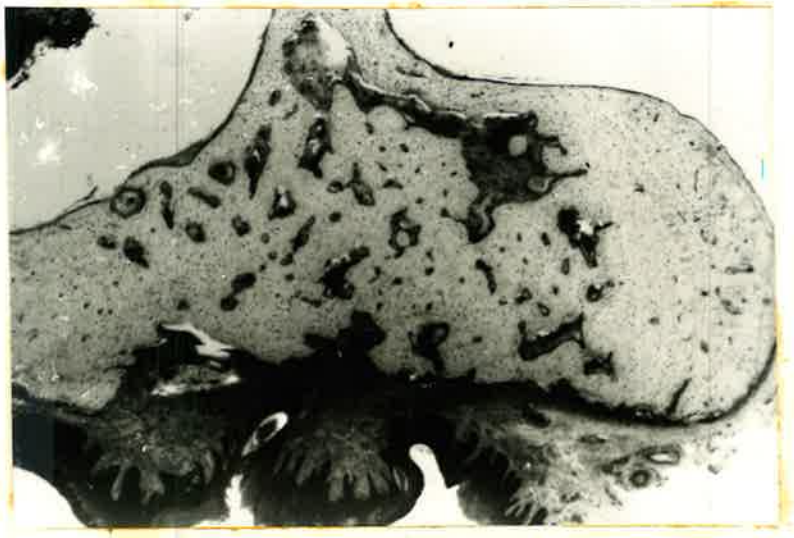


Fig. 6.102 Methylene blue X 25
8 Wks.(T/CFP.4)

General view of alvolar region. Serial section of Fig. 6.103

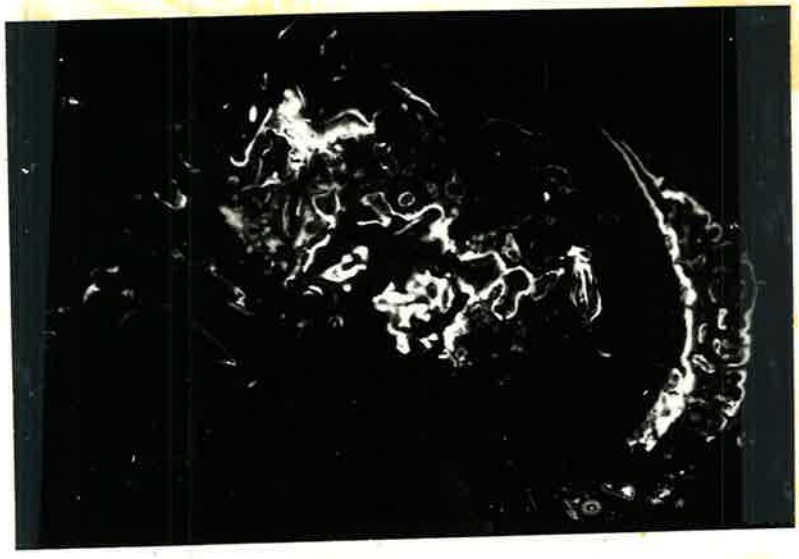


Fig. 6.103 Tetracycline X 25
8 Wks.(T/CFP.4)

Tetracycline labelling of periosteal and intra-socket bone.

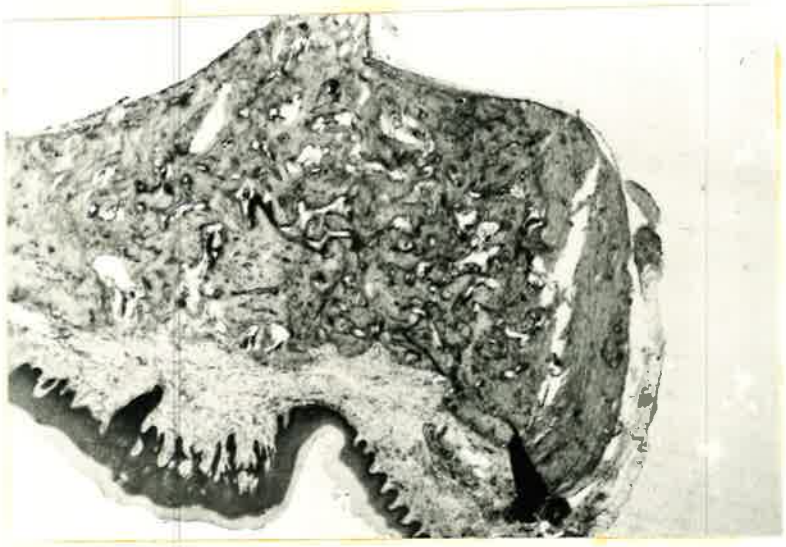


Fig. 6.104 H&E X 25 8 Wks.(T/CFP.3)

Alveolar outline.
Serial section of Fig. 6.105

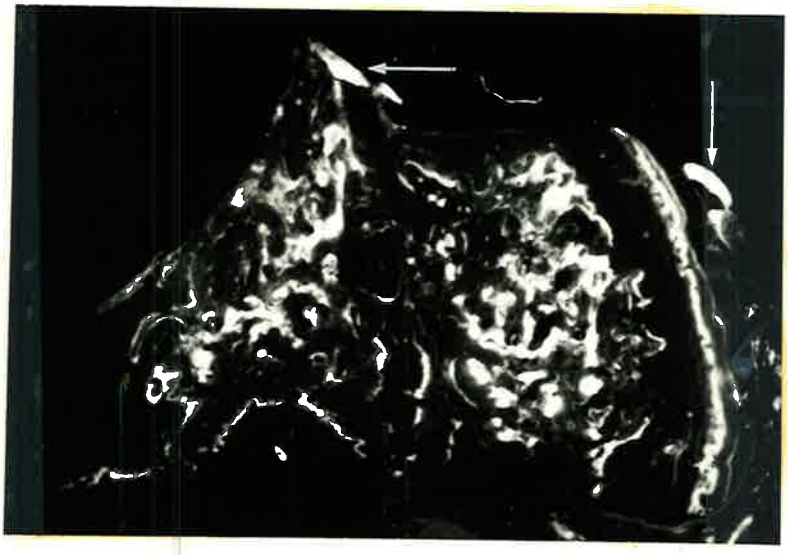


Fig. 6.105 Tetracycline X 25
8 Wks.(T/CFP.3)

Tetracycline labelling of periosteal and intra-socket bone. Auto-fluorescence in soft tissues (arrows).

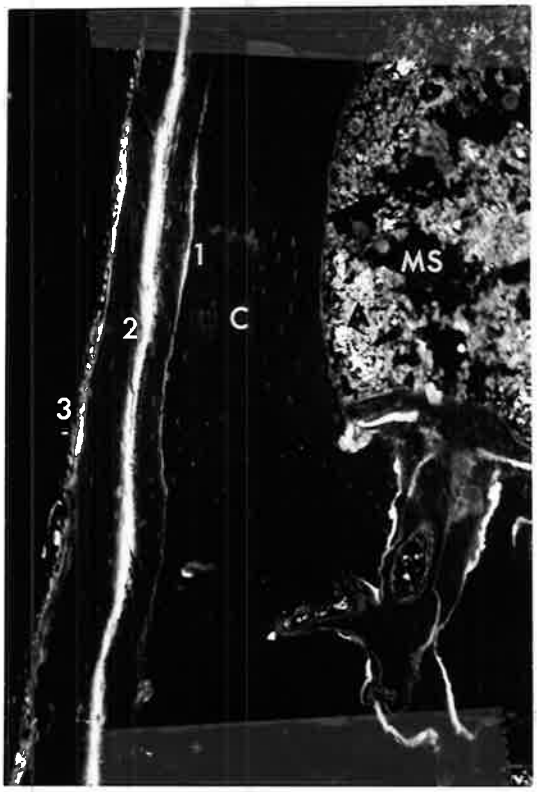
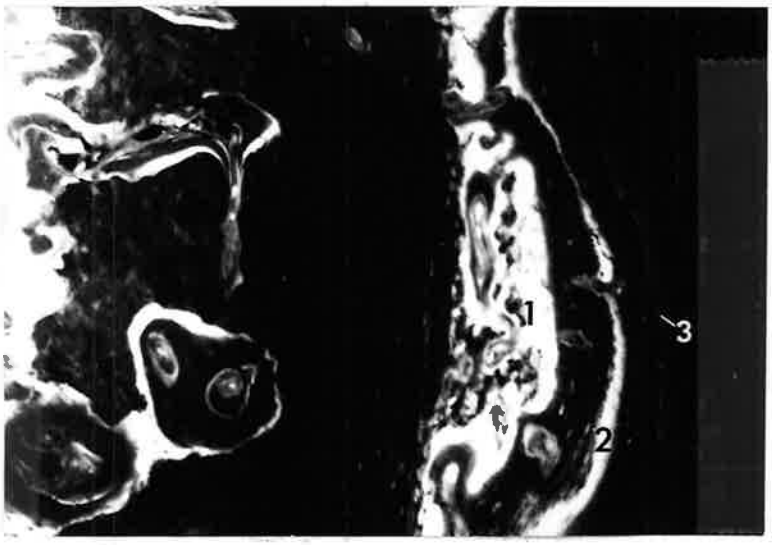


Fig. 6.106 Tetracycline X 100
8 Wks.(T/CFP.1)

Periosteal area on control side. Shows three regular tetracycline fluorescent bands (1,2,3).
Auto-fluorescence (A)
Cortical bone (C)
Marrow space (MS)

Fig. 6.107 Tetracycline X 100
8 Wks.(T/CFP.2)

Periosteal bone. Irregular first and second fluorescence bands (1,2). Third band very indistinct (3)



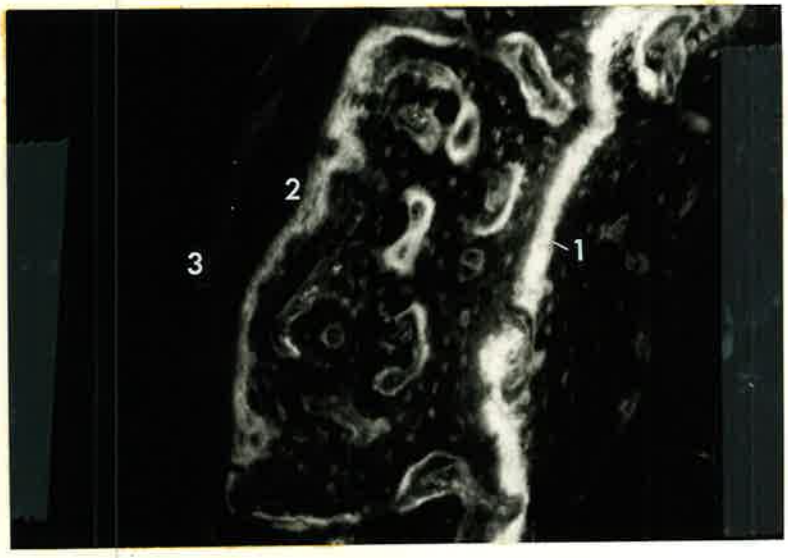


Fig. 6.108 Tetracycline X 100
8 Wks.(T/CFP.4)

Labelling bands in periosteal bone (1,2,3). Compare with Figs. 6.106 and 6.107

Fig. 6.109 Tetracycline X 40
8 Wks.(T/CFP.2)

Resorption at buccal alveolar crest. Compare with Fig. 6.100 Auto-fluorescence (arrows).

4 Months to 8 Months

At the end of 4 months, remodelling of bone had removed part of the tetracycline-fluorescence labelling (Figs. 6.111, 6.113).

At the end of 8 months, remodelling of bone had removed most of the labelled bone. However, faint outline of some fluorescence labelling could still be discerned and gave an idea of the original socket outline compared with the remodelled outline after 8 months (Figs. 6.114, 6.116).

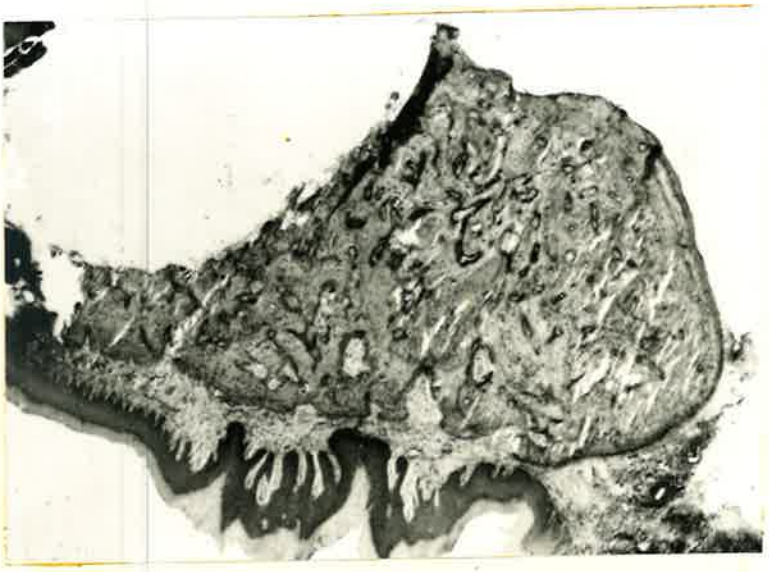


Fig. 6.110 H&E X 25 4 Mths.(T/CFP.2)

General view of alveolar region after 4 months. Serial section of Fig. 6.111.

Fig. 6.111 Tetracycline X 25
4 Mths.(T/CFP.2)

Labelled bone in socket still evident. Note weak tetracycline bands in periosteal bone (1,2) Third band is not seen

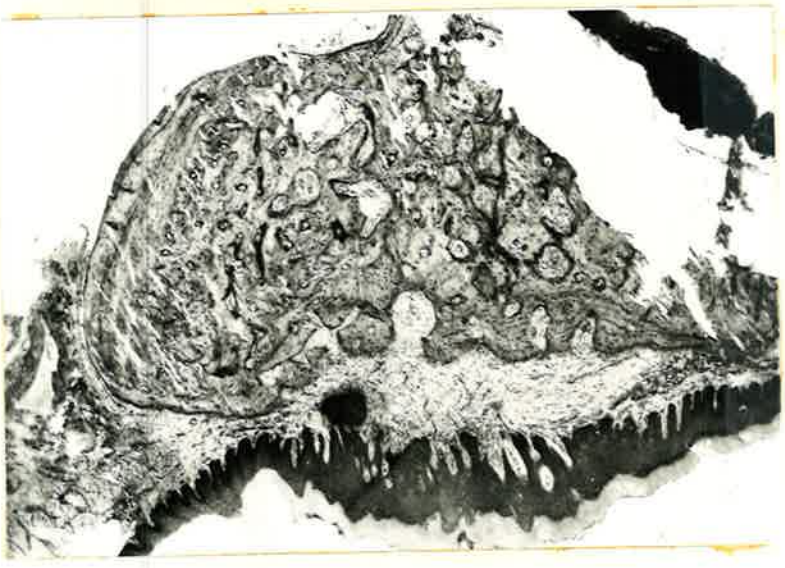


Fig. 6.112 H&E X 25 4 Mths.(T/CFP.4)

General view of socket.
Serial section of Fig. 6.113

Fig. 6.113 Tetracycline X 25
4 Mths.(T/CFP.4)

Loss of fluorescence labelling in intra-socket and periosteal bone. Artifact and auto-fluorescence (arrows).

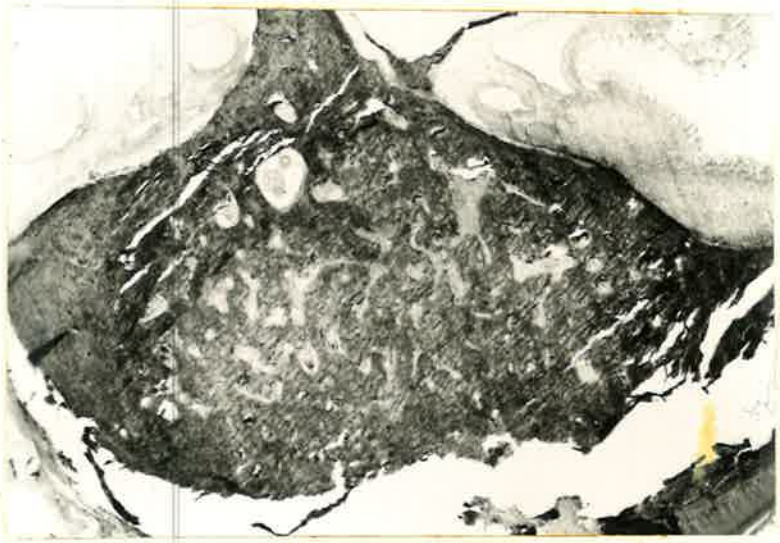


Fig. 6.114 Toluidine blue X 25
8 Mths.(T/CFP.3)

General view of alveolar region at 8 months.
Serial section of Fig. 6.115

Fig. 6.115 Tetracycline X 25
8 Mths.(T/CFP.3)

Loss of tetracycline fluorescence.
Auto-fluorescence (arrows)
Artifact (A)

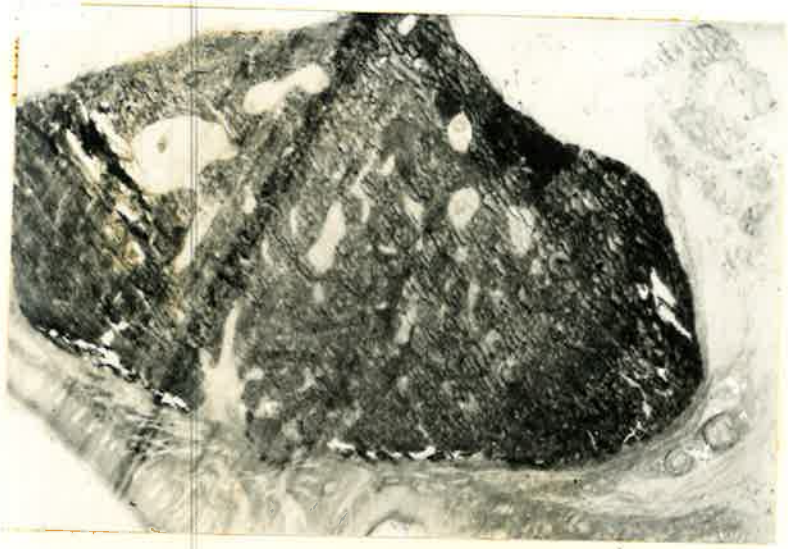


Fig. 6.116 Toluidine blue X 25
8 Mths.(T/CFP.1)

General view of alveolar region.
Serial section of Fig. 6.117

Fig. 6.117 Tetracycline X 25
8 Mths.(T/CFP.1)

Loss of tetracycline labelled material after 8 months.

lysed blood clot, which was also PAS positive, and the two were not distinguishable (Figs. 6.13, 6.22). Because of its abundance around capillaries, it seemed likely that it originated from these vessels or from the cells in close proximity to them.

At 48 hours, the PAS positive material in the interstitial spaces was not positive with Hale's stain, indicating that although it was rich in mucoproteins and glycoproteins, it did not contain demonstrable acid mucopolysaccharide.

By 72 hours, intercellular substance between the young fibroblasts of the invading granulation tissue was less strongly PAS positive, and with time, the staining declined in intensity. By this time, Hale's stain demonstrated a mild reaction by the intercellular components of the granulation tissue, indicating the presence of acid mucopolysaccharide. From this point onwards, the distribution of acid mucopolysaccharide as demonstrated by the Hale's reaction became weak and diffused. At no stage was a strongly positive Hale's reaction demonstrated in the granulation tissue within the socket. This is at variance with the observations of others (Dunphy and Udupa, 1955; Udupa and Prasad, 1963) who claimed the accumulation of mucopolysaccharide in the ground substance prior to fibrilogenesis with this build-up reaching a maximum

on the 5th post-operative day.

The negative results obtained in the present study may however, have been related partly to the small amount of acid mucopolysaccharide present, and partly to the possible loss of the material during prolonged periods of fixation and decalcification. With the dialysed iron (Hale's) method of identifying acid mucopolysaccharides, Cohen, L. (1968) has stated that ".....tissue which had been fixed for long periods in formalin and then paraffin embedded stained poorly."

Amler et al. (1964) have noted that "reticular fibres formed a fine intercrossing delicate network as the first young connective tissue cells began to invade the granulation tissue at two to three days post-operatively. These soon coalesced into larger, irregular spiralling fibre bundles which appeared to be composed of numerous fine fibrils cemented together and optically homogeneous while still retaining their argyrophilia. At the same time, these fibres gave a PAS positive reaction." This development was confirmed in the present study (Figs. 6.31, 6.54, 6.72).

Amler et al. (1964) had also noted that "reticular" fibres were heavily concentrated at the periphery of the developing bone matrix and were sparse within the matrix. An inverse

relationship between reticulin fibres and collagen fibres was also observed. These points are demonstrated in Figs. 6.51, 6.52, 6.54, 6.55.

B. The fate of the Periodontal Membrane remnants

A progressive degeneration of the collagen fibres of the remnant of the periodontal membrane was seen. This is consistent with the observations of Euler (1923); W. Meyer (1924) and Simpson (1960).

Due to early invasion by fibroblasts at 48 hours it was difficult to determine the fate of the fibrocytes in the remnant of the periodontal membrane. Although the collagen fibres degenerate and probably take no active part in the formation of granulation tissue, the fibrocytes of the periodontal membrane may not degenerate. It is possible that they might be reactivated to become functional and thus contribute to the early formation of granulation tissue.

As the technique of using colchicine to arrest mitotic division is not a precise method it cannot therefore be expected to give an accurate picture of the origin and fate of cells. This limitation therefore precludes any conclusions from being drawn regarding the eventual fate of fibrocytes in the perio-

dontal membrane remnants. The use of tritiated thymidine and auto-radiography might provide further insight into this problem.

C. Resorption of Bone in the Alveolar Socket

Although other investigators (Schram, 1929; Mangos, 1941; Simpson, 1960) observed only slight resorption of the buccal and lingual crests following extraction, Reynolds (1963), who studied the upper first molar socket in the rat, noted "widespread osteoclastic activity....with sequestration of the intraradicular and apical bone".

In the present study, extensive resorption of the alveolar socket was seen only in specimens in which there was intensive inflammation associated with food impaction and/or sequestration of dead bone and root fragments. Osteoclasts first appeared 24 hours post-operatively and became very prominent by the third day (Fig. 6.25). At the end of 5 days, resorption had progressed to such an extent that most of the bone forming the original socket had been resorbed (Fig. 6.34). Sequestration of dead bone from the buccal alveolar crest and the interseptal area was a common feature. In uncomplicated specimens the socket was replaced entirely by granulation tissue at the end of one week (Fig. 6.53). There was little inflammation in this

tissue and resorption of alveolar bone was minimal.

A likely explanation of the extensive resorption in some specimens is the amount of trauma that the alveolar socket received during the post-operative period. The diet used in this investigation was extremely fibrous and impaction of food material in the socket was a common finding despite suturing of the alveolar socket wound. Reynolds (1963) did not specify the physical nature of the diet used in his investigation so that it is not possible to say whether his results could have been due to the same causative factor or not.

Simpson (1960) noted that resorption of the buccal crest was a prominent feature. In the present study, resorption of the buccal crest was seen starting on the 14th day (Fig. 6.85) and seemed to be part of the remodelling process.

D. Osteogenesis

Before discussing the bone formed in various areas in the healing of the extraction socket, a few general remarks will first be made regarding osteogenesis.

Present knowledge indicates that osteoblasts elaborate acid mucopolysaccharide and a collagen precursor in the early stages of bone matrix formation (Chapter I). PAS stain demon-

strated in the present investigation the presence of muco-proteins and glycoproteins in the interstial areas between active osteoblasts both in the alveolar socket and in the buccal periosteum (Figs. 6.40, 6.47). The presence of acid mucopolysaccharide around osteoblasts in the socket was not conspicuous (Fig. 6.64). The limited quantity of this material present as well as a possible loss through tissue processing has been pointed out.

In the periosteal location where periosteal bone formation was excessive, the accumulation of acid mucopolysaccharide was obvious (Figs. 6.48, 6.61, 6.84). Whether this difference in intensity of staining with Hale's stain is entirely due to a quantitative difference, or whether some other factor is involved is not known.

Chlorazol fast pink and tetracycline both demonstrated areas of new bone formation. In appositional growth where the rate of bone formation was slow, these two agents produced labelling bands which coincided in distribution.

In areas where formation of bone was progressing at a rapid rate, the distribution of these two markers was different. Chlorazol fast pink stained the leading edge of the forming bone deeply (Figs. 6.42, 6.49), an area where the tetracycline

was not taken up. Presumably, this chlorazol fast pink stained area was not calcified at this stage. In areas where strong tetracycline fluorescence could be demonstrated, chlorazol fast pink staining was often faint. No explanation can be offered for this observation.

In addition to the use of demethyl-chlortetracycline, a different fluorescent bone marker, such as DCFA fluorescein, could have been used. This would have provided a better chronological localization of new bone at various post-operative periods.

E. Formation of Bone in the Socket

In the alveolar socket, trabecular bone was laid down. This was first noticed in the fundus of the alveolar socket and then on the surface of the walls. The socket was then filled in by bone in a centripetal direction. Bone was laid down either directly on to the surface of the existing socket wall or where resorption had ceased.

Boyne (1966) has critised the description by previous investigators which indicated that bone filled in from the fundus upwards towards the superficial portion of the socket. He said that this was an inaccurate observation. He pointed out that bone was formed on the walls of the socket and grew

in a centripetal manner. Serial sectioning and tetracycline labelling used in the present investigation confirmed this observation of Boyne. However, new bone was first noted in the fundus region and replacement of granulation tissue by bone progressed from the fundus region as well as from the walls of the socket.

Under normal conditions, the periodontal membrane plays a dynamic role in maintaining the attachment of the tooth to bone. There is constant deposition of cementum on the one hand and bone formation and resorption on the other. The osteogenetic capacity of cells in the periodontal membrane is therefore evident, and in this respect it resembles that of the cells of the periosteum and endosteum.

However, the fate of the cells of the periodontal membrane following extraction of the tooth could not be determined. If these cells remain viable it is conceivable that they could contribute to osteogenesis within the alveolar socket. Three other sources of osteogenic cells must, however, also be considered; first, of these could be cells released from the osteocyte lacunae following resorption of the original alveolar socket walls; secondly, cells from adjacent marrow spaces could migrate into the healing area; and thirdly induction of cells from the

granulation tissue that has filled in the socket could take place.

The colchicine technique for assessing cell division does not provide an answer to the above question.

A departure from the results of earlier investigations is the time required for bone regeneration in the socket. Huebsch et al. (1952) observed in the rat after thirteen days, that the socket was almost filled with new bone. In the present study, although the first sign of new bone in the socket appeared on the fifth day, further progress from this stage was variable. In specimens where there had not been food impaction or sequestration of root and/or bone fragments, bone replacement in the socket was well advanced by the end of the second week and was almost complete by the end of the third week. On the other hand, where inflammation persisted, the osteogenic phase within the socket was delayed (Figs. 6.62, 6.64, 6.75).

Delay in bone formation caused by the retention of root fragments has been noted by Glickman et al. (1947). However, not all retained fragments need be sequestrated and Fig. 6.69 demonstrates this. Glickman et al. (1947) also stated that roct fragments in the deeper parts of the socket need not be sequestrated.

F. Endosteal Bone Formation in Adjacent marrow spaces

Boyne (1966) has claimed that in human biopsy material, the first sign of bone formation occurred in the adjacent marrow spaces of the alveolar socket.

In the present study, however, formation of bone in the adjacent marrow spaces was of rare occurrence and took place shortly after new bone had appeared within the socket (Figs. 6.65, 6.66).

G. Formation of Periosteal Bone

The formation of bone subperiosteally outside the tooth socket is a feature which has only been recently reported (Boyne, 1962, 1963, 1966; Boyne and Kruger, 1962; Reynolds, 1963).

Reynolds wrote: "New bone was first noted on the supra-lateral aspect of the maxillae forming into the soft tissue of the cheek". The present investigation confirms this observation.

Subperiosteal activity was first noted 48 hours after extraction, on the buccal surface of the alveolar process and new bone was formed in this region by the third post-operative day. By the fifth day, the formation of periosteal bone was well

advanced and covered the entire buccal surface.

Although subperiosteal formation of bone was demonstrated on the buccal surface of the alveolus in all specimens the quantity of bone formed was extremely variable. In the healing of fractures of long bones, it has often been stated that the periosteal callus was a result of functional demand to give rigidity to the healing fracture. Pritchard (1964) has stated that the size of the callus around the injured bone is related to the instability of the bone fragments. This line of reasoning does not seem to apply in the case of the maxilla where there is no muscle action, although following the extraction of a tooth mastication might alter stress distribution on the maxillary bone.

It is speculated that periosteal bone formation observed in the present investigation is a reactive phenomenon of periosteal tissue to inflammation. In sockets which healed rapidly and without complication, the amount of subperiosteal deposit was minimal (Figs. 6.36, 6.53, 6.73, 6.76, 6.78, 6.86, 6.91). In these specimens the buccal tissues overlying the periosteum were not inflamed or if so only mildly.

On the other hand, where there was persistent inflammation in the buccal tissues, the amount of periosteal bone was

excessive (Figs. 6.33, 6.34, 6.37, 6.62, 6.67, 6.75, 6.76, 6.94, 6.96).

Two possible routes of spread of inflammation to the buccal periosteum were noted. First, inflammation could track from the margin of the buccal socket extending along the buccal surface of the periosteum. This point is substantiated by the fact that in many of the specimens showing persistent inflammation, the buccal oral mucosa had been detached and the underlying tissues exposed (Figs. 6.33, 6.34, 6.37, 6.62) or that there had been inflammation resulting from sequestration or food impaction (Figs. 6.33, 6.34, 6.62, 6.67, 6.75, 6.76, 6.93, 6.94). Trauma to the periosteal tissues at the time of operation could also account for imflammation as it could be demonstrated to occur as early as 4 hours post-operative (Figs. 6.11, 6.12).

The other possible route for spread of inflammation is via the alveolar socket wall. In all these specimens, inflammation in the buccal alveolar socket was evident, and resorption in the socket wall a prominent feature. Inflammation spreading across the alveolar socket wall to involve the buccal periosteal tissues was therefore a possibility. This received confirmation in the specimen shown in Fig. 6.64, where a periosteal deposit was formed at the entrance of a nutrient canal to

a socket which was inflamed.

However, intensive inflammation seemed to have the opposite effect of delaying bone formation. This is demonstrated in both periosteal (Figs. 6.63, 6.64, 6.37, 6.45) and intra-socket (Figs. 6.33, 6.34, 6.62, 6.64, 6.75, 6.94, 6.98) areas.

Some authors also believe that the resorption of bone released an osteogenic inductor substance (Bridges and Pritchard, 1958). If this were so, then it is also possible for this inductor substance to diffuse through the cortical bone to reach the periosteal surface, thereby inducing osteogenesis. However, in the present study, osteoclasia and inflammation in the socket were so closely associated that the independent effect of the two reactions could not be assessed.

However, both the "inflammation" and "osteogenic inductor" hypotheses do not satisfactorily explain the periosteal phenomenon observed. Both these hypotheses presume the diffusion of substances, possibly chemical in nature, reaching a bone surface. If this were so, then it would be reasonable to expect stimulation of periosteum or endosteum on all bone surfaces adjacent to the area.

In the present study, endosteal activity was seen sometimes in the adjacent marrow spaces and in Haversian systems. New bone formation on the palatal surface was not observed. Impaction of food was more frequently found in the buccal area, due to detachment of the buccal epithelium over the prominent buccal alveolar crest. The palatal alveolar crest is not prominent and the palatal mucosa is not easily detached. The epithelial covering therefore affords protection against trauma from fibrous food material. Necrosis of bone in the palatal alveolar crest was therefore not seen. However, even in cases where inflammation under the palatal mucosa was observed, no periosteal reaction was seen (Figs. 6.27, 6.34, 6.36, 6.37, 6.67, 6.76, 6.96).

Boyne and Kruger (1962) observed new bone formed on the floor of the antrum opposite to the alveolar socket. No such reaction was seen on the nasal surface of the maxillary-complex in this study.

Yet another question must be asked in relation to periosteal bone formation. Until 1962, no investigator had reported this phenomenon, and a close scrutiny of material presented by the various authors prior to this time failed to reveal evidence of it. The photomicrographs which give

adequate coverage to permit examination of periosteal surfaces include material from dogs (Claflin, 1936), man (Schram, 1929), rats (Huebsch et al. 1952) and monkeys, (Simpson, 1960)

In 1962, Boyne observed that "....a study of mandibular extraction wound healing in dogs demonstrated the existence of new bone formation in areas adjacent to the healing sockets proper. These regions of bone formation were observed: (1) subperiosteally along the lingual cortex; (2) overlying the mandibular canal; and (3) in crestal bone areas." This was followed by Reynold's report on rats which has already been referred to.

This periosteal bone formation is not a unique phenomenon of rat tissue as it has also been observed in the dog (Boyne, 1962, 1963; Boyne and Kruger, 1962) and in man (Boyne, 1966). The same phenomenon was observed by the author in guinea-pigs in a preliminary investigation (unpublished).

The author believes that the phenomenon is related to the presence of inflammation in the buccal tissues and in the socket area, caused by the impaction of coarse food particles or the sequestration of necrotic bone and small root fragments.

Boyne (1963) has also noted that when a gross amount of alveolar bone had been removed at the time of operation, subperiosteal deposition was greatly increased. This increase could be accounted for by the increase in inflammation produced by the surgical trauma induced.

It is unfortunate that the nature of the diet used in many of the investigations was not given thereby not making it possible to determine whether the lack of post-operative trauma resulting from the use of a soft diet was responsible for the absence of this phenomenon of periosteal bone formation.

II. CORRELATION OF HEALING IN FRACTURES, CORTICAL DEFECTS AND IN THE EXTRACTION WOUND.

From the results of the present investigation and the discussion, certain general principles in the reaction of bone to trauma may be formulated. With different types of injury osseous repair follows essentially the same stages which are:-

(i) The formation of a haematoma,

(ii) Organization of the haematoma by granulation tissue,

(iii) Periosteal reaction,

(iv) Endosteal reaction,

(v) Formation of new bone to replace the granulation tissue bridging the area of injury,

(vi) Remodelling.

The formation of a haematoma, the inflammatory reaction following tissue injury and the organization of the haematoma were consistent features which were observed by all investigators in the healing of fractures, cortical defects and in the extraction wound. These phases of wound healing therefore require no further discussion.

A. Periosteal Reaction

Periosteal reaction is a consistently observed phenomenon in the healing of fractures. It has been noted that the quantity of periosteal callus formation is related to the degree of apposition and fixation of the bone fragments. In accurately apposed and rigidly fixed fragments, the formation of periosteal callus is minimal. Kuntcher (1967) further believes that the extensive periosteal callus formed in unstable fractures is related to the amount of inflammation around the fracture area brought on by constant mechanical trauma to the wound (page 2.13).

In the healing of defects in cortical bone, formation of periosteal callus is also a consistent feature (Bast, Sullivan and Geist, 1925; Melcher and Irving, 1962, 1964).

It is significant that Melcher and Dreyer (1962) have reported the formation of periosteal bone on the surface of the femur opposite to the defect (pp.2.19; 2.20). Although these authors attributed this phenomenon to an alteration of functional forces transmitted through the bone in that area, this periosteal reaction could have been brought about by spread of inflammation.

Recent reports and the results of the present investigation also show that in the healing of the extraction wound, periosteal reaction is present. The results of the present investigation suggest that the quantity of periosteal bone formed may be related to the persistence of inflammation in the healing site. This supports the view that periosteal reaction is a reaction of bone to trauma and that the amount and duration of inflammation in the area of injury determines the quantitative aspect of this reaction.

B. Endosteal Reaction

Endosteal reaction leading to the formation of endosteal callus is a pronounced feature in the healing of fractures. In investigating the healing of drill-holes in the cortical bone of the rat femur, Melcher and Dreyer (1962) noted that extensive callus was often formed on the endosteal surface

adjacent to the drill-hole and sometimes on the endosteal surface directly opposite the cortical defect. They believed that this osteogenic reaction might be attributed to trauma induced in the endosteum of that area.

In both healing in fractures and in cortical defects, the marrow spaces are directly involved in the initial trauma. The endosteal surfaces in these cases are therefore directly involved by the inflammatory reaction that follows. If inflammation were to serve as a direct stimulus to endosteal reaction, it would therefore follow that endosteal callus formation in these types of injury would be pronounced.

In the extraction wound, the adjacent marrow spaces are not directly involved. These spaces are separated from the wound by a layer of alveolar bone. Inflammation therefore does not primarily involve these marrow spaces. However, the diffusion of inflammatory exudate across the thin alveolar walls is possible. If this were so, it would explain the relatively mild endosteal reaction seen in the marrow spaces adjacent to the alveolar socket as observed in the present investigation and by Boyne (1962, 1966).

C. Osseous repair of injured bone

The third phase of osteogenesis in the reaction of bone

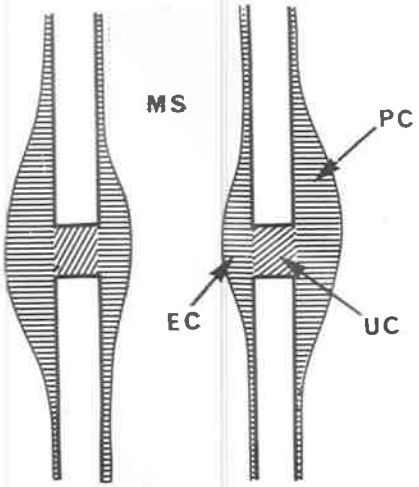
to trauma is the formation of new bone to bridge the site of injury. In the healing of a fracture this has been termed the uniting callus (page 2.7) and is the last portion of the ossifying callus complex to become mineralized (Urist and McLean, 1941 a & b; Ham and Harris, 1956; Nilsonne, 1959). In the healing of a cortical defect, the filling in of the defect area by new bone was also the last phase of osteogenesis (Melcher and Dryer, 1962). In the present investigation of the healing of the alveolar socket, filling in of the socket by new bone also came after the periosteal reaction had started.

D. Remodelling

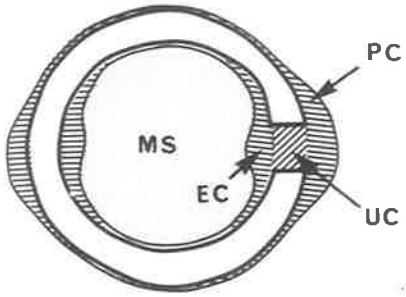
The final phase in the healing of osseous tissue is the remodelling of the new bone formed. In the healing of fractures and cortical defects, the remodelling process resulted in the resorption of a large portion of the periosteal and endosteal callus. What remained of the callus was remodelled and lamellar bone laid down. In the present investigation, resorption and remodelling was also noted.

Thus it can be stated that the process of healing following fractures, perforating wounds in cortical bone and the extraction of teeth follow essentially similar lines. The principles

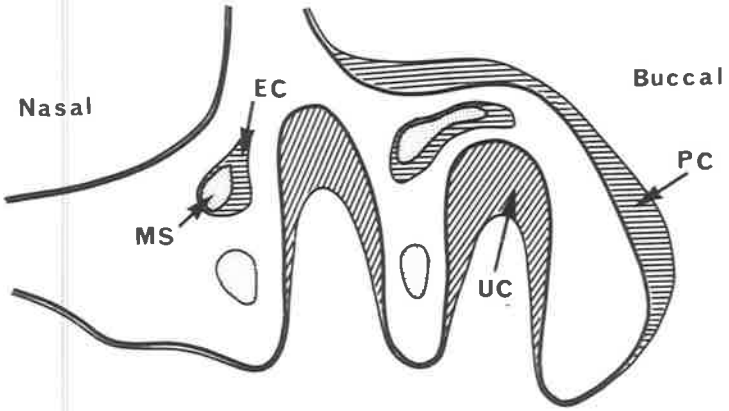
of tissue reaction to trauma in all three situations are the same. Minor differences relating to time sequence and the relative quantity of each reacting component, for example periosteal bone, endosteal bone and replacement bone are probably the result of local factors influencing each of these components to various degrees under the differing circumstances. Fig. 7.1 gives a diagrammatic representation of the comparison and correlation between fracture healing, healing in cortical defects and in the extraction wound.



I. FRACTURE



II. CORTICAL DEFECT



III. ALVEOLAR SOCKET

Fig. 7.1

Healing in Fractures, Cortical Defects and Alveolar Socket.

PC Periosteal Callus. UC Uniting Callus. EC Endosteal Callus.
MS Marrow Space.

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

An experimental project has been carried out designed to investigate healing in the alveolar socket of the maxillary molar in the rat.

Histological and histochemical methods have provided information regarding the histogenesis of various tissue components in the healing wound. The results obtained from these methods have confirmed the findings of previous investigators in the formation of granulation tissue, intercellular fibres and bone matrix.

The present study has also confirmed that the remnants of the periodontal fibres degenerate following tooth-extraction and take no part in repair process. The fate of the cells in the periodontal remnant however cannot be determined.

The degree of resorption of the alveolar socket walls was found to be dependent on the amount of inflammation present in the socket. Intensive inflammation was also found to delay healing in the alveolar socket, both at the stage of proliferation of granulation tissues and in its subsequent replacement of bone. The persistence of inflam-

mation was due to the impaction of foreign materials and sequestration of dead bone and/or root fragments.

The pattern of osteogenesis in the healing alveolus conforms to the general pattern of osseous repair in other conditions such as fractures and cortical defects. An attempt has been made to correlate the reaction of various tissue components as seen in the above types of bone injury.

Inflammation is believed to be the primary causative factor in stimulating periosteal and endosteal reactions. Its persistence determines the amount of bone formed by these tissues.

The formation of the uniting callus and bone in the alveolar socket is the result of the replacement of granulation tissue. The source of osteogenetic cells in this location could not however be determined with the techniques used in the present investigation.

The combined use of chlorazol fast pink and demethylchlortetracycline labelling has made it possible to determine the areas of bone matrix formation and its subsequent mineralization in the different areas of osteogenesis.

APPENDIX I

THE RAT DENTITION

The dentition of the rat is monophyodont. It consists of one incisor and three molars in each quadrant of the jaw (Fig. App. I.1). The incisors have persistent pulps. The molars are of limited growth and resemble somewhat minute human molars (Fig. App. I.2). Their development is chiefly limited to the first 40 days of life (Schour and Massler, 1942). Secondary cementum in the first molar begins to form about the 35th day post-partum and is concomitant with the functional stresses imposed upo n the teeth. This is added continuously throughout the life of the animal, so that in the older animals, one third or more of the relatively long root can be cementum only (Schour and Massler, 1943). The roots are 3 to 5 in number (Table App. I.1).

Table App. I.1 Anatomy of the molars of Rats
(From Schour and Massler, 1942)

Number of Cusps	M-1 $\frac{5}{5}$	M-2 $\frac{4}{4}$	M-3 $\frac{3}{3}$
Number of roots	M-1 $\frac{5}{5}$	M-2 $\frac{4}{4}$	M-3 $\frac{3}{3}$
Secondary cementum Forms 1/3 or more of root			

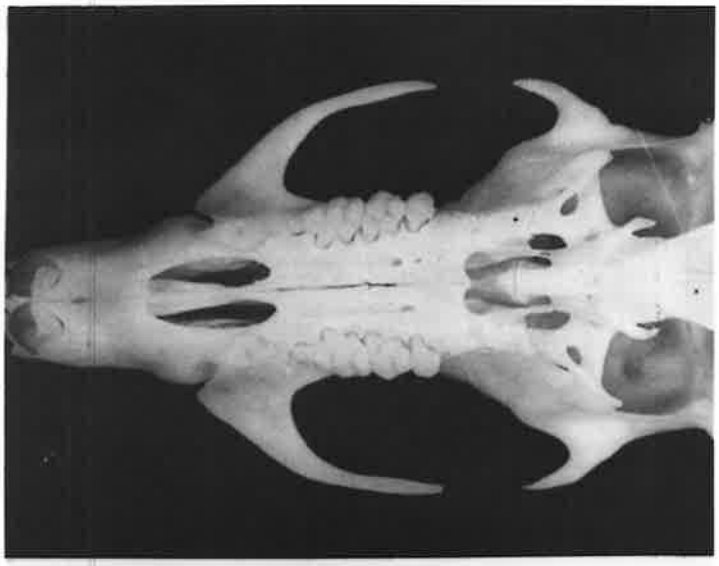


Fig. App.I.1 BASAL VIEW OF RAT SKULL

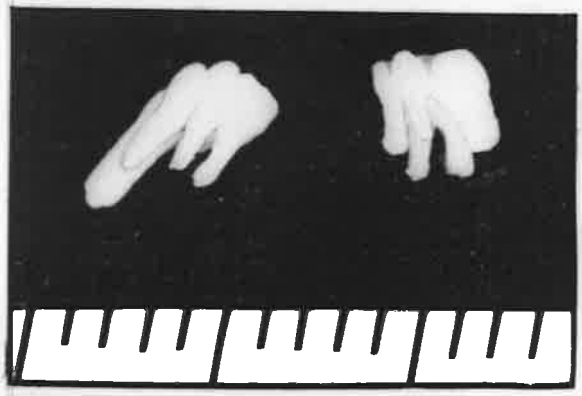


Fig. App.I.2 UPPER FIRST MOLARS OF THE RAT
BUCCAL AND PALATAL VIEWS
SCALE IN MILLIMETERS

APPENDIX II

DIET

Except for the post-operative 24 hour period, animals used in the investigation were fed on the following stock diet, provided by Thomas H. Webb Pty. Ltd., Wingfield, S.A. 5013.

Special Mouse, Rat and Monkey Diet M 164

Ground Wheat	40.0%
Ground Barley	18.0%
Bran and Pollard	12.0%
Meat and Bone Meal	9.6%
Ext. Soya Meal	6.2%
Fish Meal	6.2%
Milk Powder	3.0%
Brewer's Yeast	1.0%
Salt	1.0%
Molasses	3.0%

Vitamin Supplement per Kilogram of Feed

Vitamin A_3	3928 I.U.
Vitamin D_2	928 I.U.
Vitamin B_6	1.5 mg.

Vitamin B_{12} (Pyridoxine hydrochloride)	0.2 mg.
Vitamin B	3.4.mc gms
Vitamin E	1.2 mg
Vitamin K (Menadione)	0.5 mg.
Panthothenic Acid	0.5 mg.
Choline Chloride	25 mg.

APPENDIX III

OPERATIVE INSTRUMENTS AND PROCEDURES

The instruments required for extraction of teeth in rats consist of the following:-

A modified dental spoon-excavator,

A fine probe,

A small metal spatula (cheek guard),

A pair of 5 in. scissors.

5-0 braided black silk sutures,

5/8 cleft palate suture needle,

Mosquito artery clips, straight (needle holder),

A pair of College tweezers.

A plastic operating stand was constructed, with an attachment for maintaining jaw-opening. Initially, the procedure was performed with the aid of a dissecting microscope with a light source for illumination of the field (Fig. App. III.1.). With increase in skill, the microscopic aid was not necessary, and illumination was provided by wearing a Storz 15-watt head-light. The operative field was kept dry by using a tiny glass sucker attached to a Venturi pump (Fig. App. III.1). Figs. App. III.2 and App. III.3 show the operative procedures.

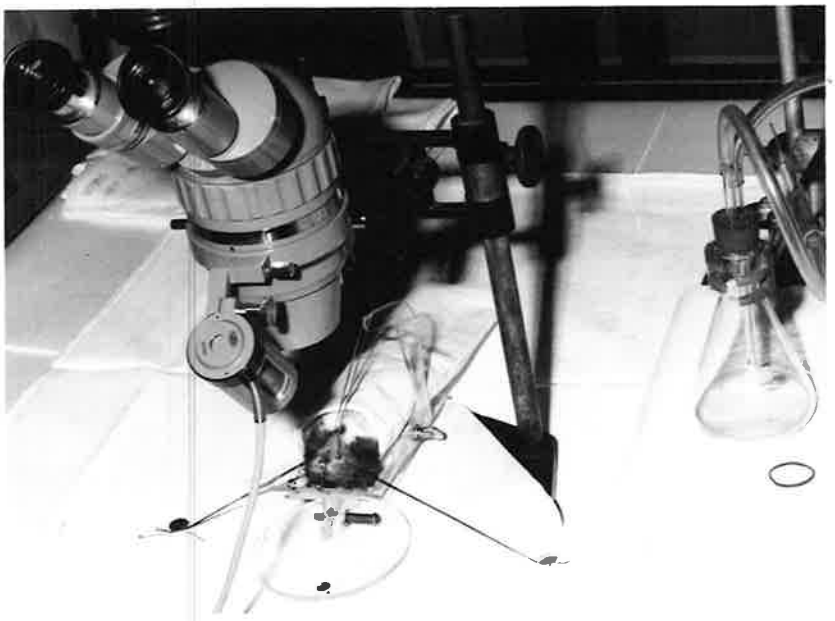


Fig. App.III.1 SET-UP FOR OPERATION

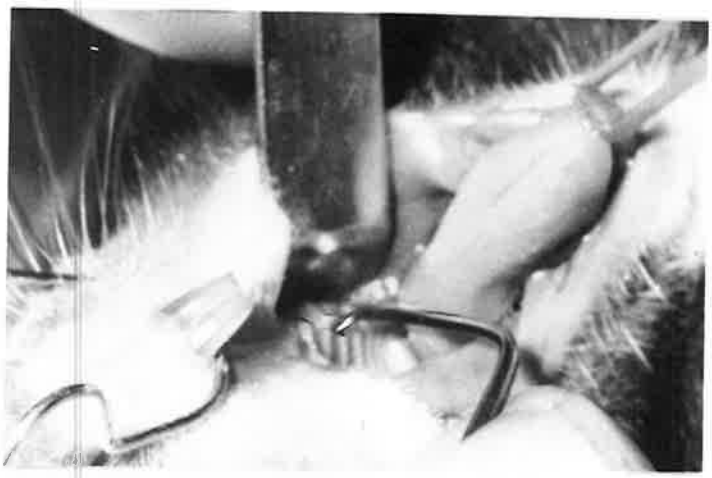


Fig. App.III.2 SEPARATION OF GINGIVAL ATTACHMENT

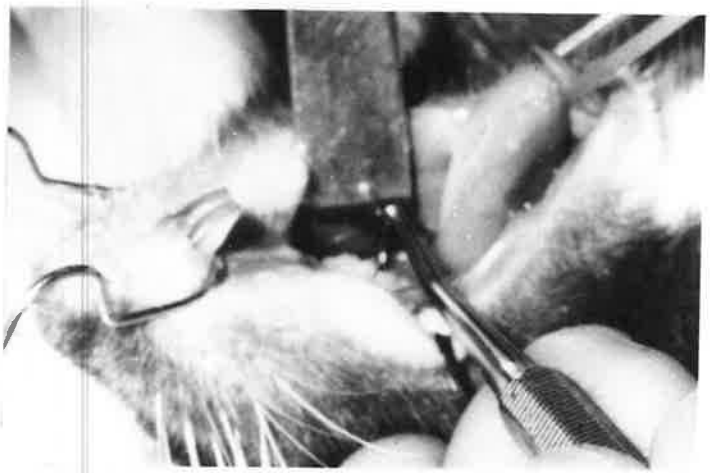


Fig. App.III.3 LUXATION OF THE TOOTH

APPENDIX IV

PREPARATION OF DRUGS FOR INJECTION

DEMETHYL-CHLOR-TETRACYCLINE

A commercial preparation, Ledermycin (150 mg. capsules) has been used. These were dissolved in 0.9% sterile saline to give a concentration of 10 mg. per ml.

Storey (1965+, 1968) has recommended a dosage of 50 mg. per kg. body weight for rats.

CHLORAZOL FAST PINK

The dye (Matheson, Coleman and Bell, BX 340) was dissolved in sterile 0.9% saline to make a 5% aqueous solution.

Modall (1939) has used a dosage of 12.5 to 50 mg. per 100 gm. body weight in cats. Moss (1954) gave mice three intra-peritoneal injections at half-hourly intervals, each injection of 0.5 ml. of a 5% solution, the total dosage given being 75 mg.

Storey (1968) recommended a dose level of 25 mg. per kg. body weight for 2 days. This appears a rather low dose level as compared with the previously mentioned authors. In the present investigation, a dose of 25 mg. per 100 gm. body

+ Personal communications

weight given on 2 consecutive days has been used.

COLCHICINE

Supplied by the British Drug Houses Ltd., Poole, England. Crystals were dissolved in 0.9% saline to make a 0.02% (20 mg. per 100 ml.)

APPENDIX V

ULTRA-VIOLET FLUORESCENCE MICROSCOPY AND PHOTOMICROSCOPY

Leitz[#] fluorescence equipment was used. Ultra-violet light source was provided by a HBO 200 W high pressure mercury vapour lamp. As the absorption band for tetracyclines is from 420 milli-microns to 460 milli-microns (Frost et al. 1961), the following combination of filters were used as recommended by the manufacturers:-

Primary Filter : 5 mm. BG 12

Secondary Filter : Blue absorption (K 530)

The following optical system has been found satisfactory:-

Bright field condenser: Leitz No. Code 600, Numerical aperture 0.25, sometimes in conjunction with condenser top with aspherical lens giving a Numerical aperture of 0.90.

Objectives:	Achromatic	2.5/0.07
	Plano	4/0.10
	Plano	10/0.25
	Achromatic	25/0.50
	Plano	40/0.65
Eye-piece:	x 10	

Leitz --- Ernst Leitz GMBH, Wetzlar, Germany.

An alternative system using Phase Contrast equipment with condenser after Heine (refer Leitz List 513-5c/Engl.) was also used. This equipment offered the possibility of examination in phase contrast, bright field, and dark field with continuous transition between these types of illumination. It was therefore useful for examination of unstained sections and for correlation of tetracycline and chlorazol fast pink labelling in the same section.

Phase ccntrast condenser after Heine : Leitz No. 64

Objectives: Achromatic Pv 10/0.25 n
.Achromatic Pv 25/0.50 n

Fluorescence photomicroscopy was carried out with the Leitz Orthomat microscope camera equipment (refer Leitz List 54-19a,b/Engl.). Photoflure Orthochromatic# film was used and reproduced on Ilfobrom+ IB 5 photographic paper.

Photoflure Orthochromatic film (ASA 400), Kodak (Australasia) Pty. Ltd., Melbourne, Australia.

+ Ilfobrom paper, Ilford (Australia) Ltd., Melbourne, Australia.

APPENDIX VI

PREPARATION AND SECTIONING OF UNDECALCIFIED BONE SPECIMENS

The histological preparation of undecalcified bone has not been very successful until quite recently. The main obstacle being the inability to cut thin sections of this hard structure.

Ground sections by manual methods, for example, that of Frost (1958), provide fairly good material for micro-radiographic studies and for ultra-violet fluorescence microscopy. However, soft tissues are not preserved and consequently, the relationship between soft tissue and bone cannot be ascertained. The thickness of sections made by this method cannot be accurately controlled, and serial sections cannot be prepared.

With the introduction of methacrylate embedding for ultra-microtomy (Newman, Borysko and Swerdlow, 1949) several attempts have been reported in which undecalcified bone specimens were embedded in plastic materials and sectioned with a microtome (Woodruff and Norris, 1955; Bohatirchuk, 1957; Kuhn and Lutz, 1958; Yaeger, 1958; Ueckert, 1960).

These methods have the following advantages: (i) the relationship between hard and soft tissues is preserved, (ii) thin sections of 6 to 8 microns can be produced, and are therefore of optimal thickness for histological examination, (iii) serial sectioning can readily be accomplished, (iv) most routine histological stains can be applied to these sections, and (v) sections are also suitable for ultra-violet fluoresce microscopy.

The Method of Ueckert (1960) has been used for the preparation of undecalcified specimens in this study.

Attempts at duplicating this technique were met with difficulties, particularly in the adequate infiltration and even polymerisation of the polyester resin in bone. Different types of commercial polyester resins and plastizer were tried and eventually, the following modified schedule was found to be satisfactory.

DEHYDRATION

Alcohol 70%)	
Alcohol 80%)	3 hours in each
Alcohol 90%)	
Alcohol 95%)	
Alcohol Abs.)	6 hours in each
Alcohol Abs.)	
Alcohol Abs.	overnight

CLEARING

Methyl benzoate -- 2 changes, 6 hours each
Methyl benzoate -- 24 hours
Chloroform -- 2 changes, 3 hours each

INFILTRATION

Polyester[#] / chloroform in equal parts, plus catalyst[+] 0.2% -- 3 changes, 24 hours each.
Mixture kept at low temperature in refrigerator.

VACUUM IMPREGNATION

- Fresh polyester/chloroform/catalyst mixture, and chloroform evaporated under reduced pressure (28 - 30 in.Hg.) at 40°C until volume was reduced by one third.

- Polyester/chloroform in 2 : 3 ratio plus catalyst 0.2% mixture kept at low temperature overnight.

- Fresh mixture as above and remainder of chloroform evaporated under reduced pressure at 40°C.

- Undiluted polyester and 0.5% catalyst, kept at low temperature for 2 days.

Polyester -- Crystic 191 Low viscosity or Crystic 141 S.P. Monsanto Chemicals.

+ Catalyst -- Methylethyl ketone peroxide

BLOCKING

Polyester	9 parts
Plasticizer (Di-butyl-phthalate)	1 part
Catalyst	0.5%

Specimens were set in correct alignment in plastic moulds, blocked, and allowed to polymerise at 40°C.

TRIMMING

Polymerised blocks were trimmed to correct shape and size (Fig. App. VI.1), with the cutting surface less than 1 cm. in width, and tapered in front.

Sections were cut on a heavy duty sledge microtome, using a wide-back bi-planar knife (Ueckert, 1960), and with diluted detergent (Teepol) as a lubricant.

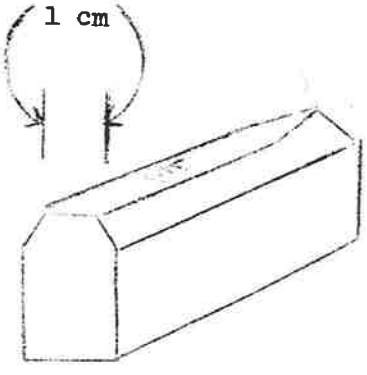


Fig. App. VI.1 Trimmed polyester resin block

APPENDIX VII

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

FIXATION

Fixation was in buffered neutral Formol-Saline 10% for 24 - 48 hours.

Formal Saline

Formaldehyde (40%)	100 ml.
Sodium chloride	9 gm.
Distilled water	900 ml.

The solution was buffered with magnesium carbonate to excess.

DECALCIFICATION

Formic Formate Solution

Sodium Formate	68 gms.
Distilled water	1660 c.c.
Formic Acid	340 c.c.

Specimens were immersed in 20 times their volume of this solution, and changed everyday until decalcified. The degree of decalcification was checked by radiographic examination.

DOUBLE EMBEDDING TECHNIQUE

Following decalcification, the specimens were passed through:

1. 5% sodium sulphate -- 8 hours for neutralization
2. 80% ethyl alcohol at 37°C -- 12 hours
3. 85% ethyl alcohol at 37°C -- ½ hour
4. 90% ethyl alcohol at 37°C -- ½ hour
5. 95% ethyl alcohol at 37°C -- ½ hour
6. 3 changes of absolute alcohol at 37°C for 1 hour each change
7. Methyl salicylate, abs. alc. equal parts for 1 hour
8. Methyl salicylate at 37°C -- 2 hours
9. 0.5% celloidin in methyl salicylate -- 2 hours
10. 1% celloidin in methyl salicylate -- 3 to 4 days
11. 1 part paraffin wax (M.P.58°C) and 2 parts methyl salicylate at 60°C -- 1 hour
12. Equal parts of paraffin wax and methyl salicylate at 60°C -- 1 hour
13. Two parts of paraffin wax and one part of methyl salicylate at 60°C -- 1 hour
14. 3 changes of paraffin wax (M.P.58°C), the last change being put under increasing vacuum to 25 in. Hg. -- 24 hours in all
15. The specimens were then blocked.

HAEMATOXYLIN AND EOSIN STAIN, VAN GIESON'S STAIN AND MALLORY'S ANILINE BLUE STAIN

Procedures used for these stains were from Manual of Histologic and Special Staining Techniques, A.F.I.P. (1960), pages 29, 62 and 60 respectively.

SILVER STAINING.

(Lillie's Silver Oxide Method, 1946, modified 1954)

(Lillie, R.D., Histopathologic Technics and Practical Histochemistry, 3rd Edition, 1965, pages 528 and 536)

1. Deparaffinised in xylol, then through absolute alcohol, 90% alcchol, 70% alcohol.
2. Washed in tap water for 1 minute
3. 0.5% potassium permanganate --- 2 minutes.
   190 ml. of 0.5% pot. perm.
   10 ml. of 3% sulphuric acid — Made up just before use.
4. Washed in tap water
5. Treated with 5% oxalic acid --- 5 minutes
6. Washed in tap water
7. Applied ferric chloride --- 2 minutes
8. Washed in tap water for 3 minutes
9. Washed in de-ionised water --- 2 changes
10. Immersed in diammoniacal silver nitrate solution --- 3 minutes
11. Drained and rinsed quickly in distilled water
12. Flooded with 10% formalin for 1 - 2 minutes --- till section turning black
13. Washed in running water for 3 minutes
14. Toned in 2% acid gold chloride for 2 minutes
15. Rinsed in water
16. Fixed in 5% sodium thiosulphate for 2 minutes
17. Washed in water

18. Dehydrated in 2 changes of absolute alcohol

19 Cleared in xylol

20. Mounted in Caneda balsam

Diammoniacal silver nitrate solution

1 ml. Concentrate ammonium hydroxide in small flask
Titrated with 10% silver nitrate until faint permanent tupidity remained.

Diluted with equal parts of distilled water

PERIODIC ACID - SCHIFF'S STAIN (McMANUS, 1948) for GLYCOPROTEINS AND MUCOPROTEINS. (After DIASTASE).

(Barka & Anderson, Histochemistry, 1963, Page 80).

1. Deparaffinised in xylol, then through absolute alcohol, 70% alcohol, and to water.
2. Diastase for 30 minutes.
3. 0.5% Periodic acid --- 10 minutes
4. Rinsed in tap water 5 - 10 minutes
5. Rinsed in distilled water --- 3 minutes
6. Schiff's reagent --- 30-45 minutes
7. Rinsed in three changes of sulphurous acid --- 2 minutes for each rinse.
8. Washed in tap water --- 2 minutes
9. Washed in distilled water --- 5 minutes
10. Counter-stained with Mayer's haematoxylin
11. Washed in tap water for 10 minutes
12. Dehydrated, cleared and mounted

A control section of umbilical cord tissue was used

A second control section was also used without Diastase

With some sections, no counter-stain was used and steps 10 and 11 were omitted.

DIALYSED COLLOIDAL IRON ABSORPTION STAIN (HALE'S REACTION) FOR ACID MUCOPOLYSACCHARIDES

1. Section deparaffinised in xylol, then through absolute alcohol, 70% alcohol and to water

2. Section stained for 2 hours in the following solution:-

Stock colloidal iron solution	10 ml.
Glacial acetic acid	12 ml.
Distilled water	18 ml.

3. Rinsed in 3 changes of 30% acetic acid, 10 minutes each

4. Washed in running tap water for 5 minutes, then rinsed in de-ionized distilled water

5. Immersed for 20 minutes in the following freshly prepared solution:

2% potassium ferrocyanide solution	1 part
2% hydrochloric acid	1 part

6. Rinsed for 5 minutes in running tap water, then in distilled water briefly

7. Counter-stained with 1% neutral red for 1 minute

8. Rinsed in distilled water to remove excess stain.

9. Dehydrated, cleared and mounted

With some sections, no counter-stain was used and steps 7 and 8 were omitted.

Preparation of the stock colloidal iron solution was carried out as suggested by Mowry* (1963), Practical Histochemistry, Page 512:-

Iron chloride solution --- 5.46 gm. of $FeCl_3.6H_2O$ in 10 ml. of distilled water.

The above solution was added to 250 ml. of boiling distilled water, 250 ml., and boiled until solution became dark red.

After cooling, free acid and unhydrolysed iron salts were removed by dialysis for 24 hours.
The solution was then filtered.

The working colloidal iron solution was prepared fresh before use as follows:-

Distilled water	18 ml.
Glacial acetic acid	12 ml.
Stock colloidal iron solution	10 ml.

* Mowry, R. (1963)

The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. Ann. N.Y. Acad. Sci., 106:402

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