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A COMPARISON OF DIFFERENT HISTOCHEMICAL TECHNIQUES TO TRACE BONE MINERAL DEPOSITION

by

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A COMPARISON OF DIFFERENT HISTOCHEMICAL TECHNIQUES TO TRACE BONE MINERAL DEPOSITION

Abstract

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Recent years have seen a tremendous growth in interest in the noncollagenous proteins of bone extracellular matrices as specific markers of bone cell populations, modulators of matrix mineralization and calcium homeostasis, and possible local controlling and coupling factors of normal bone turnover. Preliminary investigations have indicated that serum albumin is present in the calcified bone matrix and may be associated with the mineralization front during enamel and dentin formation.

Albumin, a plasma protein, is present in extravascular sites in all connective tissues which have been investigated. However, the amount of albumin and the kinetics of exchange between circulating and tissue albumin vary from one tissue to another. Albumin may therefore play different roles in different tissues; yet little is known about its localization and possible function in any calcified tissue.

The purpose of this study was to investigate the localization of albumin in bone tissue as a step towards understanding the significance of its presence. In addition the

localization pattern of albumin was compared to that of two commonly used fluorochrome bone markers, tetracycline and (2,4-bis[N,N'-dicarbomethyl] amino ethyl) fluorescein (DCAF), to elicit its potential role as a tracer of bone formation.

Laterally directed force was applied to the maxillary central incisors of young male Sprague Dawley rats by means of a standardized intraoral coil spring inserted under constricting pressure (15g) into holes drilled in the rats' two maxillary central incisors. Control animals had either a passive appliance placed or no appliance at all. Experimental animals received one of the following injections: intravenous bovine serum albumin (BSA), intraperitoneal or intramuscular tetracycline, or intraperitoneal DCAF. Control animals received intravenous injections of phosphate buffered saline (PBS).

At the end of the three day experimental period, maxillary central incisors and surrounding bone and mid-palatal suture were surgically removed and prepared for histologic processing. Half of the specimens were decalcified in EDTA before embedding. BSA sections were stained by an indirect immunoperoxidase technique. In addition, endogenous rat albumin was localized by similar methods. Tetracycline and DCAF sections were observed under ultraviolet light.

The investigation demonstrated a simple procedure that provided a good anatomical model for bone remodeling in response to sutural separation. Findings showed light, non-specific

background staining for BSA sections regardless of decalcification. Endogenous rat albumin was present ubiquitously. Undecalcified tetracycline and DCAF sections showed appropriate fluorescence at the mineralization front, while demineralized sections were devoid of any fluorescence.

The results of this study in regard to immunostaining are inconclusive and suggest that the technique for localization of exogenous serum albumin is in need of refinement. Tetracycline and DCAF are effective bone tracers in undecalcified specimens. Results from fluorescent labeling correlated well with routine histologic findings and showed greater bone deposition on the suture side versus the periodontal membrane side of the same bone in response to an activated appliance.

DEDICATED

TO

Chang Ho and Sookhi Kim, my parents, who encouraged me to make my dreams become reality,

and

Carol Lynn Kim, my wife, whose never-ending love and devotion provided the energy needed to go on when I felt I could not.

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INTRODUCTION

While the phenomenon of bone remodeling is readily observable, the biochemical processes involved are largely unknown. Protein biochemistry has long been a center of interest for many investigators, but only recently have the noncollagenous proteins of the extracellular matrix of bone attracted much attention.

Of the noncollagenous proteins, serum albumin is but one of many possible factors involved in bone remodeling. Its function of binding and transporting calcium along with its importance in the stabilization of the regulatory phase in mineralization has led to much speculation in regard to its true role in this process.

Albumin is present in extravascular sites in all connective tissues which have been investigated. However, the amount of albumin and the kinetics of exchange between circulating and tissue albumin vary from one tissue to another. Albumin may therefore play different roles in different tissues; yet little is known about its localization and possible function in calcified tissue.

Thus, one of the purposes of this study was to investigate the localization of albumin in bone tissue as a step towards understanding the significance of its presence. A better understanding of the factors responsible for the initiation, rate

and termination of bone remodeling may lead to improved management of orthopedic redirection of growth patterns during orthodontic treatment of facial disharmonies and improve the range and stability of possible tooth movements while decreasing iatrogenic damage to the roots and periodontium.

Preliminary studies have indicated that serum albumin is present in the calcified bone matrix and may be associated with the mineralization process at the mineralization front during dentin and enamel formation. Thus, a second purpose of this study was to compare the localization of albumin to that of two commonly used fluorochrome bone markers, tetracycline and DCAF, to elicit its potential role as a tracer of bone formation.

BACKGROUND

Craniofacial growth and development is a dynamic, multifactorial phenomenon involving displacement and remodeling. The bones of the craniofacial complex do not merely increase in size, but also change in shape and position during growth and development as determined by functional and genetic factors. Any given bone grows in response to functional relationships established by the sum of all the soft tissues operating in association with that bone. Thus, the bone itself does not regulate the rate and directions of its own growth; the functional soft tissue matrix is the actual governing determinant of the skeletal growth process. This functional matrix concept is fundamental toward understanding the nature of a bone's role in the overall growth control process (Moss and Young, 1960; Enlow, 1982).

Thus, the functional matrix concept may account, at least in part, for the mechanical force that carries out the displacement process. Still controversial though is the impact of intrinsic genetic influence. While established and valid, the functional matrix concept describes essentially what happens during growth. It is not intended to explain how the growth control mechanism actually functions and does not account for the regulatory processes at the cellular and molecular levels that carry it out (Enlow, 1982).

The formation and remodeling of bone is precisely regulated in space and time. Cellularly, the process of bone remodeling is mediated by osteoblasts and osteoclasts. These represent fully differentiated cells of finite lifespan that require continuous renewal (Parfitt, 1984). One can postulate the existence of progenitor cell populations as has been suggested for the histologically similar periodontal membrane (Melcher, 1976; Gould et al., 1977; Gould, 1983). Although controversial, it is held that these cells represent derivatives of separate cell lines (Peck, 1979; Parfitt, 1984).

Bone resorption, mediated by the osteoclast, involves removal of both mineral and organic intercellular substance. Ham (1974) suggests that osteoclasts function by providing a local acidic environment. Thus, calcium phosphate solubility increases and is eroded. Osteoclastic enzymes then degrade the organic matrix. In addition, mononuclear cells have been proposed to accomplish collagen digestion (Heersche, 1978).

In bone deposition, matrix precursors are secreted by osteoblast cells and form bone matrix with the onset of osteoblastic extracellular enzymatic action (Ham, 1974; Shapiro, 1981). The cells can influence the initiation of mineralization by controlling the concentration of inorganic ions. Possible control points include intracellularly at the mitochodrial level, extracellularly via matrix vesicles (Anderson, 1976; Shapiro, 1981), or by osteoblast-derived macromolecules (Termine et al.,

1981). Osteoid will calcify with progressive impregnation by calcium phosphate salts. The mechanism for calcification is complex and not fully understood.

Physical stress may have a direct action on bone growth as has been demonstrated for bone in tissue culture (Glucksman, 1938, 1942). Orthodontic tooth movement, a convenient model for the bone remodeling process, involves a force delivery system designed to treat malocclusions. The forces delivered by orthodontic appliances are transferred to the periodontal membrane and the alveolar process housing the teeth. The classic hypothesis on the mechanism of tooth movement postulates the generation of pressure and tension sides within the periodontal space when the tooth is translated through the alveolar bone (Reitan, 1960, 1964). Compression of the periodontal membrane produced on the pressure side induced osteoclastic activity resulting in bone resorption. Simultaneously, the collagen fibers are stretched on the tension side. This results in the formation of new bone by osteoblast activation (Reitan, 1960, 1964). This hypothesis restricts the tissue changes incident to tooth movement to the tooth socket. With the exception of the periodontal membrane, the alveolar process represents a static structure through which teeth translate during orthodontic treatment.

Investigation by Baumrind (1969) led to the first departure from the classic pressure-tension hypothesis. His findings

indicate that plastic deformation of the alveolar process accounts for the majority of early tooth movement. Moreover, this bone deflection occurred before the compression of the periodontal membrane. DeAngelis (1970) further disputed the pressure-tension hypothesis in a radioisotope study showing localization patterns in areas other than the tension side of the periodontal membrane. These findings led him to believe that a remodeling process similar to the mechanism of craniofacial growth was also occurring in the alveolar bone involved in orthodontic tooth movement. This drift process of bone remodeling was originally described by Enlow (1968). Additionally, he postulated that the plastic deformation of the alveolar process incident to orthodontic tooth movement may create a piezoelectric effect. This in turn will alter the electric environment and stimulate bone progenitor cell differentiation resulting in bone remodeling as well as tooth movement.

Rodan et al. (1975 a,b) were first to show mechanical stress induced change in ionic fluxes. Thus, the possibility exists for mechanical force conversion to electric current before a cellular response. Electric charge polarization, referred to as piezoelectric effect has been described previously (Fukada and Yasuda, 1957; Bassett and Becker, 1962). Numerous investigators have demonstrated the application of low-level electric currents to bone in vivo directly (Bassett et al., 1964; Lavine et al.,

1972; Brighton et al., 1977) or indirectly (Davidovitch et al., 1980 a,b; Finkelson et al., 1983) results in cell activation and bone turnover. In addition, accelerated tooth movement was observed when exogenous electric current was administered in conjunction with orthodontic force (Davidovitch et al., 1980). While bone formation occurs in electronegative regions, bone resorption predominates in electropositive areas (Pawluk and Bassett, 1970; Zengo et al., 1973).

The factors and events required for the initiation of the cellular response of bone remodeling are still unclear. The cyclic nucleotides have been identified as mediators for effects on bone cells produced by external stimuli in vitro (Rodan et al., 1975; Kornstein et al., 1981; Norton et al., 1977) and in vivo (Davidovitch et al., 1977, 1978; Nagata, et al., 1975). Davidovitch and Shanfeld (1975) demonstrated higher concentrations of cyclic AMP (cAMP) in alveolar bone extracts from orthodontically treated cats versus untreated controls and Binderman et al. (1974) produced increased cAMP levels in bone cells with physical strain on cultured condyle cells. Fluctuations in cAMP levels were observed in the periodontal membrane when subjected to mechanical force (Davidovitch et al., 1978) and a combination of mechanical stress and electric current (Davidovitch et al., 1980, 1984; Kornstein et al., 1981; Binderman et al., 1984). It has been postulated that cAMP may act as a second messenger to some hypothetical first stage

chemical mediator occurring as a result of mechanical and electrical stimulations (King and Theims, 1979; Mostafa et al., 1983). However, little is known about the first stage mediator as well as the sequence of events spanning between the increased level of cAMP and actual bone resorption and deposition.

The prostaglandins have long been implicated as mediators in bone remodeling and have been postulated to interact with cAMP during the local control process. PGE_2 has been shown by many investigators to increase cAMP in bone (Yu et al., 1976; Somjen et al., 1980; Binderman et al., 1982, 1984; Dziak et al., 1983). Prostaglandins stimulate bone resorption in vitro (Klein and Praisz, 1970; Tashjion et al., 1977; Graves et al., 1980; Dewhirst, 1984) and in vivo (Goddson et al., 1974). Dziak et al. (1983) demonstrated direct evidence for the existence of specific PGE_2 receptors in bone. In addition, Yamasake et al. reported acceleration of orthodontic tooth movement by prostaglandin in rat (1980), monkey (1982), and human (1984). Although prostaglandin effects have been widely studied, the nature of the relationship among applied mechanical stress, prostaglandins, and cAMP has not been conclusively shown.

The biochemical events leading to mineral formation is obscure. Biomineralization is a complex phenomenon involving the interaction of inorganic as well as organic phases (Veis and Sabsay, 1983). The components of the organic matrix in calcified tissues serve either a structural role of supporting and

orienting the mineral phase or are actively involved in initiation and regulation of mineral deposition. Recent years have seen a tremendous growth in interest in the noncollagenous proteins (NCP) of bone extracellular matrices as specific markers of bone cell populations, modulators of matrix mineralization and calcium homeostasis, and possible local controlling and coupling factors of normal bone turnover. Delmas et al. (1984) identified 40 protein groups from EDTA extracted bovine bone with two-dimensional gel electrophoresis. Among these, 15 were not present on plasma maps and most of their identities are still largely unknown. Thus, the NCPs of bone are poorly characterized despite their potential importance in the development, maturation, and physiology of the tissue. There have been indications that a spectrum of NCPs exist, but to date, very few of them have been purified or characterized.

Neuman et al. (1982) presented evidence that NCPs stabilized brushite in solution as well as in the presence of apatite. They suggest this brushite-apatite-bone protein system exhibits solubility characteristics that can resolve the old problems presented by the participation of the skeleton in extracellular calcium homeostasis on one hand, and by the apparent insolubility of the apatite mineral of bone on the other.

Several classes of NCPs are synthesized in bone. The major NCPs synthesized by bone cells and unique to bone include bone phosphoproteins, osteonectin, osteocalcin or bone-Gla protein,

bone proteoglycan, bone morphogenetic protein, bone sialoprotein, and bone proteolipid.

Bone-Gla protein or osteocalcin (OC), a 49 residue protein, has been isolated and is characterized by three residues of the vitamin K-dependent calcium-binding amino acid γ-carboxyglutamic acid. OC is synthesized de novo in bone tissue (Lian and Friedman, 1978; Nishimoto and Price, 1979) and recent studies suggest that the 1-25-dihydroxy vitamin D_3 metabolite modulates its synthesis (Lian et al., 1982 a, b; Price and Baukol, 1981; Gundberg et al., 1983).

The function of OC is unclear. OC has been demonstrated as a chemoattractant for mononuclear cells (Malone et al., 1982; Mundy and Poser, 1983) and bone depleted of OC is poorly resorbed (Lian et al., 1984). As a result of these preliminary studies, a role for OC in the mechanism of bone remodeling has been postulated. Some of the properties of OC such as binding to hydroxyapatite (Hauschka and Carr, 1982), its concomitant appearance with bone formation (Hauschka and Reid, 1978), and the constant molar ratio of OC to collagen and calcium in bone (Lian et al., 1982c) indicate that OC synthesis may also be related to bone formation and mineralization.

Despite early optimism in regard to the significance of OC, subsequent studies have shown OC to inhibit mineral accretion and crystal proliferation by surface-mediated effects (Boskey et al., 1985). Moreover, it appears to have little affinity for the

initially formed small crystals or for membrane components thought to be involved in the initiation of hydroxyapatite formation. In light of these more recent investigations, OC may actually have little effect in mineral initiation (Boskey et al., 1985). OC may play a role in regulating bone resorption instead of controlling bone apatite crystal growth.

Termine et al. (1981 a,b) have reported the isolation of three proteins from bovine bone that bind to the mineralized matrix. Of these, osteonectin (ON), a 34k dalton protein, was shown to bind collagen and hydroxyapatite. They also reported that ON may be unique to bone as ON appears to be a marker for mature matrix-producing osteoblasts by virtue of its localization to bone trabeculae and newly formed osteoid. Its role in the initiation of mineral deposition has been speculated.

Subsequent studies have obscurred the role of ON. Tung et al. (1985) and Wasi et al (1984) identified ON in periodontal membrane, and a protein with similar properties was found to be synthesized by fibroblasts. In addition, studies by Otsuka et al. (1984) have shown a decreased ability by porcine ON to bind collagen. Further investigation is necessary to define the role of ON.

Phosphoproteins inhibit in vitro hydroxyapatite formation and may play a regulatory role in mineral crystal orientation and growth (Menanteau et al., 1982; Termine et al., 1981; Veis et al., 1977). Generally, phosphoprotein concentrations increase at

the mineralization front and at sites of ectopic calcification. In addition, certain phosphoproteins extracted from mineralized tissue can promote hydroxyapatite formation from metastable calcium phosphate solutions (Boskey, 1981). Since there are several phosphoproteins in the mineralized tissues, it is possible that these proteins could serve several purposes, such as orienting the mineral, regulating the rate of mineral growth, and/or initiating mineralization (Boskey and Posner, 1984).

Bone proteolipid appears to play a direct role in mineral formation. An integral part of bone cell membranes, bone proteolipid and its component calcium-acidic phospholipid-PO_4 complexes (Boyan-Salyers and Boskey, 1980) cause in vitro and in vivo bone formation (Boskey and Posner, 1984). At present, it is thought that these complexed acidic phospholipids form on cell or matrix vesicle membranes prior to mineral deposition and provide a site on these membranes for the initiation of mineral deposition (Wuthier, 1982).

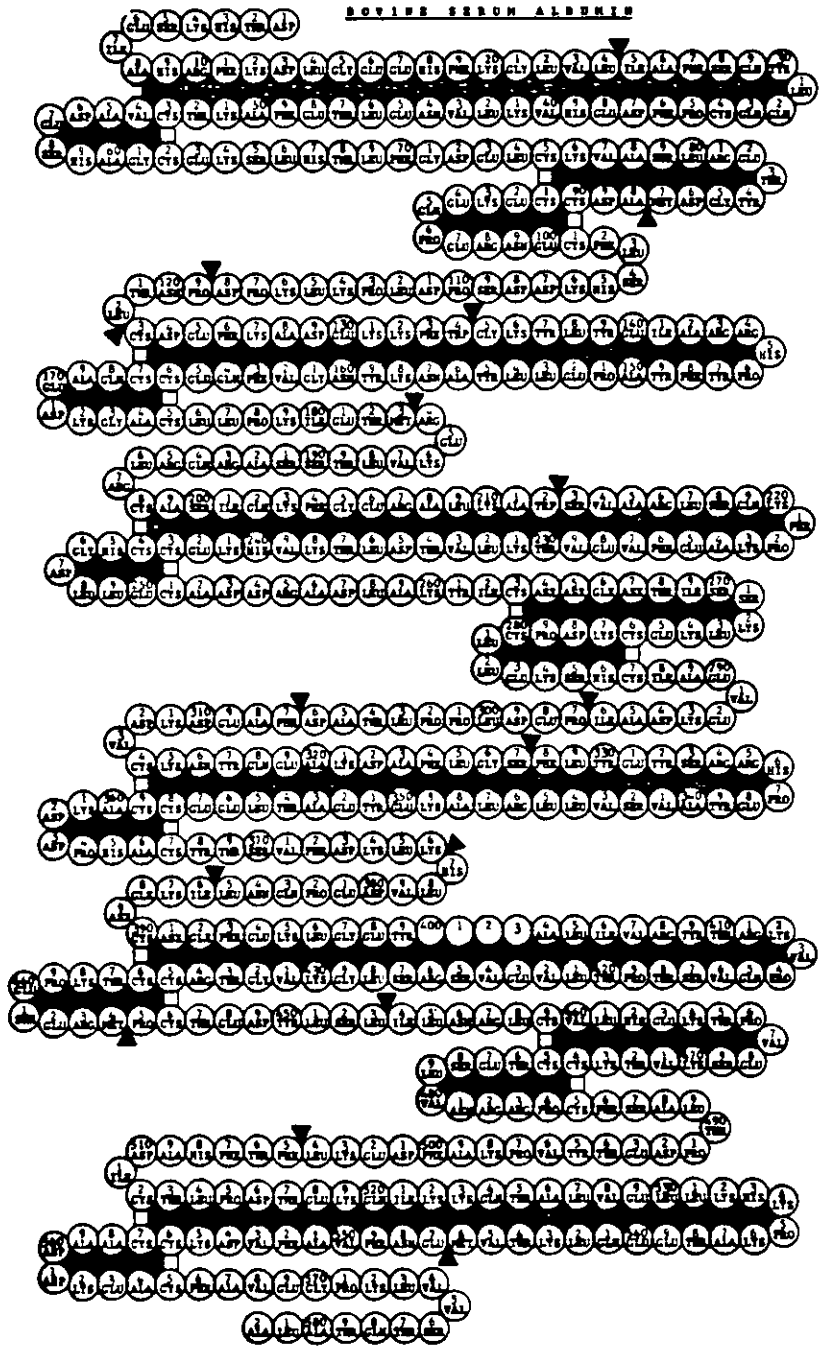
Bone morphogenetic protein (BMP) is a glycoprotein present in low concentrations in bone. First isolated and characterized by Urist et al. (1979), it is this protein that is believed to be responsible for the osteoinductive properties of demineralized bone matrix (Urist et al., 1982, 1983). BMP appears to cause a mitotic invasion of cells that eventually form bone in extraskeletal tissues. The mechanism by which BMP causes recruitment and activation of bone-forming cells is not known.

The noncollagenous matrix of bone contains proteins derived from plasma as well as protein synthesized locally (Herring, 1977; Ashton et al., 1976). Four plasma proteins, albumin, α1 acid glycoprotein, and α_2MS glycoprotein and IgE, have been shown to be specifically concentrated in bone from plasma, although the mechanism of this concentration is unclear (Mbuyi et al., 1982; Triffitt and Owen, 1977; Triffitt et al., 1976, 1978). Other plasma proteins such as IgG appear to be associated with blood vessels of bone (Ashton et al., 1976).

Serum albumin, a 582-residue protein (Fig. 1), is a versatile, rugged protein with functions so numerous that it is difficult to name one as primary. As the major protein produced in the liver and chief extracellular circulating protein, albumin is important in maintaining the circulation. As the solubilizing agent for long chain fatty acids, it is essential to the metabolism of lipids. Albumin is life-saving through its binding of toxins and through its binding of bilirubin in the jaundiced newborn. Its breakdown provides amino acids for nutrition of peripheral tissues (Peters and Reed, 1977; Rothschild et al., 1977).

Albumin and other plasma proteins are present in bone not only within the blood vessels and surrounding interstitium, but also in the bone tissue fluid and trapped within the calcified matrix (Lipp, 1967; Owen and Triffitt, 1972, 1976; Owen et al., 1973). Direct tracing experiments with fluorochrome-labeled

Figure 1. Amino acid sequence and disulfide bridges of bovine serum albumin. (Peters and Reed, 1977)


BOVINE SERUM ALBUMIN

homologous blood serum show that certain serum components are taken up by the bone substance of young and adult rats. These proteins are concentrated and incorporated into the organic matrix while it is being formed (Lipp, 1967). Fractionation experiments have identified albumin and other plasma proteins in extracts of bovine and rabbit bone (Lipp, 1967; Ashton et al., 1974; Triffitt and Owen, 1973).

Iodinated albumin was used to investigate the anatomical distribution of albumin in bone tissue (Owen et al., 1973). The results show that albumin is present in bone on at least two counts: (1) it is a transient component of the extravascular tissue fluid of bone and (2) it is a permanent constituent of calcified bone matrix, where it is incorporated during bone formation. Albumin extracted from bone tissue fluid and from calcified matrix has been shown to be immunologically identical to serum albumin.

In the matrices of dentin and enamel of rabbits, the incorporation pattern of homologous serum albumin was investigated by an autoradiographic method (Kinoshita, 1979). In addition, immunochemical experiments have been concerned with the presence of serum in the non-collagenous matrices of human dentin and bone (Ashton et al., 1976, Thomas and Leaver, 1975). Okamura (1983) was first to study the localization of serum albumin in dentin and enamel by immunohistochemical methods. Experimental results showed that serum albumin was incorporated into the

calcified matrix at the mineralization front.

Preliminary results of Owen et al. (1973) indicate serum albumin may also be associated with the mineralization front during bone formation. However, the immunolocalization of serum albumin in response to bone remodeling has not yet been studied.

The localization of albumin at the mineralization front may be related to the concentration of calcium there, as albumin has a function of binding and transporting calcium (Manuta et al., 1957, Owen et al., 1973). In addition, it is considered that albumin is important in the stabilization of the regulatory phase in mineralization, since Blumenthal et al. (1975) showed that the conversion of amorphous calcium phosphate to crystalline hydroxyapatite is slowed down in the presence of serum proteins.

Although the art and science of orthodontics has been in existence for over 50 years, knowledge of the mediators and biochemical processes involved in bone remodeling subsequent to orthodontic and orthopedic forces and craniofacial growth and development is obscure. In the last 20 years there has been a flourishing of research in the area of local control mechanisms involved in growth and remodeling. To date a definitive answer has not been found. A better understanding of the factors responsible for the initiation, rate and termination of bone remodeling may lead to improved management of orthopedic redirection of growth patterns during orthodontic treatment of facial disharmonies and improve the range and stability of

possible tooth movements while decreasing iatrogenic damage to the roots and periodontium.

Histological and cytological methods employing vital staining or chemical techniques have been used in combination with optical methods for over a century to study the nature of the tissue and cellular composition of the skeletal system, its growth, development and remodeling. In the investigation of mineralized tissues, radioactive tracers have been used extensively in biochemical studies of bone. Autoradiographic studies have come into wide use (Tonna, 1979).

While truly dynamic methods of studying cellular kinetics have been introduced with the advent of radioactive tracers and autoradiographic technology, great caution must be exercised in handling the bone-seeking radiotracers. At the tissue level, the non-specific nature of radioisotope labeling techniques, such as those using tritiated amino acids, represents the biggest drawback. Some techniques, such as those involving alkaline earth radiotracers, require the preparation of undecalcified sections of bone, which are more difficult to section than decalcified tissues and are consequently thicker. In addition, an inefficient autoradiographic technique must be employed. Even with high resolution methods an overall efficiency of only 2% is claimed, limiting microscopic observation and severely restricting any quantitative analysis (Tonna, 1979).

The study of bone has largely been undertaken with the use

of nonradioactive markers before the advent of radioactive tracers. These markers include a wide variety of dyes exhibiting numerous chemical and physical properties, including fluorescence.

Since Milch et al., (1957, 1958) introduced the use of tetracycline as an in vivo tracer of bone formation, the tetracycline method has become the most popular method to date. In a properly planned experiment, giving due consideration to the time of tetracycline administration and animal sacrifice time, numerous parameters can be obtained, namely: total bone formation, total matrix formation rate, periosteal bone deposition rate, periosteal matrix deposition rate, mineralization lag time, osteoid maturation rate, periosteal initial mineralization rate, endosteal bone resorption rate, and linear rate of endosteal bone resorption (Baylink et al., 1970).

The unstable nature of tetracycline bands under UV light precludes any quantitative analysis of band intensities. Prolonged exposure to UV light results in a decrease in fluorescence. Furthermore, since undecalcified sections are necessary, it is difficult to regulate section thickness rendering band intensity measurements questionable (Tonna et al., 1984). Study of undecalcified specimens usually involves the use of ground sections or a high-speed bone mill. In either case, cellular preservation is sacrificed.

Prolonged and large doses of oral tetracyclines can

adversely affect the normal intestinal flora of the subject under study and thus indirectly influence the experimental results (Frost, 1969). Tetracyclines have been reported to inhibit the process of mineralization (Bevelander et al., 1960; Rolle and Bevelander, 1966), disturb enamel formation (Omnell et al., 1970), interfere with bone ossification in organ culture (Saxen, 1966; Kaitilia, 1971), and also inhibit the conversion of apatite from the amorphous phase to the crystalline phase (West and Storey, 1972). Tetracycline toxicity has been detailed previously (Yen and Shaw, 1972, 1974, 1975 a,b).

Interest in fluoresceins (DCAF) as bone and dental markers has been largely directed to their use in combination with other fluorescent dyes to generate polychromatic banding (Suzuki and Matthews, 1966; Modis, et al., 1969; Rahn and Perren, 1970, 1971; Cleall, 1974; Hammond and Storey, 1974; Rahn, 1978). DCAF has been used successfully as a bone and dental marker in dogs (Modis et al., 1969; Sullivan, 1972), guinea pigs (Soni and Malloy, 1976), rats (Hammond and Storey, 1974), and rabbits and mice (Suzuki and Matthews, 1966).

Caution towards possible toxicity must be exerted with the use of DCAF at levels 30mg/kg body weight and above (Tonna et al., 1984). Color emission of bone may differ from treated apatite and fresh bone samples. Embedding materials, histological processing and increased section thickness due to the necessity of undecalcified sections also affect the final

spectral emission and intensity. Furthermore, fluorescence may fade rapidly and consequently photomicrography must be performed as soon as possible to obtain maximum color intensity.

Immunohistochemical methods utilizing specific antibody are powerful techniques in studying the relative distribution, localization, and secretory pathways of the macromolecules in the connective tissue. Radioisotope studies have confirmed the presence of albumin in calcified bone matrix. In addition, albumin has been associated with mineralization in dentin and enamel as shown by immunofluorescence. Thus, the significance of assessing the potential of serum albumin as a bone tracer by immunolocalization becomes apparant. Routine histologic processing could be used and decalcification would not hinder its localization (Okamura, 1983). Moreover, the potential toxicity and adverse growth affects of the flourochromes vital dyes or radiotracers may be avoided.

STATEMENT OF THESIS

Recently, the noncollagenous proteins of bone extracellular matrices have attracted much attention in regard to their potential importance in bone remodeling. Albumin, a plasma protein, is present in extravascular sites in all connective tissues which have been investigated. Much research has dealt with its presence in the calcified matrices of dentin and enamel, including autoradiographic and immunochemical studies. A recent investigation has shown that serum albumin may be associated with the mineralization process at the mineralization front during dentin and enamel formation.

Studies involving serum albumin and bone have been less definitive. Initial experiments using direct fluorochrome labeling confirmed the presence of a variety of plasma proteins in bone. However, no attempt was made to discern albumin from other plasma proteins. Subsequent studies have identified albumin in normal and pathologic bone extracts using immunodiffusion and radioisotope techniques. However, the distribution or pattern of localization of albumin could not be interpreted by these techniques.

Iodinated albumin was used to investigate its incorporation in calcified bone matrix. The study confirmed the presence of albumin in bone tissue fluid and calcified matrix. However, the

model utilized in the autoradiographic investigation was unsuitable for accurate descriptive analysis of the deposition pattern of albumin. In addition, less specific autoradiographic techniques were employed. Moreover, potentially adverse affects on cell activity and bone deposition due to excessive radiation can be speculated.

Immunohistochemical methods utilizing specific antibody are powerful techniques in studying the relative distribution, localization and secretory pathways of the macromolecules in connective tissues such as bone. The indirect immunoperoxidase technique proposed in this study may elicit the localization of albumin in bone tissue as a step towards understanding the significance of its presence.

In addition, the localization pattern of albumin was compared to that of two commonly used fluorochrome bone markers, tetracycline and DCAF. If the localization pattern of albumin during bone deposition is similar to that seen during dentin and enamel formation, albumin may have potential as a bone tracer. Thus, adverse effects on tissues observed from radioisotope studies or vital bone markers would be avoided. In addition, the technique would enable greater ease of handling specimens and allow for more diversity through use of decalcified or undecalcified tissues.

METHODS AND MATERIALS

Animals

The study utilized 20 young, growing, male Sprague Dawley rats. The rats were ordered to weigh 175-200 gms on delivery from Zivic Miller Laboratories (Allison Park, Pennsylvania). Rats weighing less than this were too small for the surgical intravenous injection procedure.

The rats were used immediately upon arrival if possible with a maximum lag period of three days. The animals were weighed on the first experimental day and at the time of sacrifice.

Each cage (17"x13"x8") housed four animals in a temperature (23-25^{o}C) and humidity (10-20%) controlled environment. The rats were fed a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, Missouri) and tap water ad libitum.

Experimental Design

The procedure used to apply laterally directed force to the rats' maxillary central incisors was similar to that used by Stark and Sinclair (1987). My study utilized 20 growing, male Sprague Dawley rats. At the start of the experiment, rats were approximately 40 days old, as determined by weight, and were sacrificed three days later. The animals were equally divided into five groups: control, BSA, two tetracycline groups, and DCAF. Each of these groups were further divided equally into

two subgroups based on activation of an intraoral coil spring. The control group received an intravenous injection of phosphate buffered saline (PBS). The BSA group received an intravenous administration of bovine serum albumin (BSA) while the tetracycline groups received both intraperitoneal and intramuscular injections of tetracycline respectively. The DCAF group was injected with DCAF intraperitoneally (Table I).

The injections were performed immediately after placement of the intraoral coil spring. The dosage per kilogram body weight were as follows: BSA IV 1.5 g, Tetracycline IP and IM 30 mg, and DCAF IM 15 mg. A modified procedure utilizing a surgical technique was employed for intravenous injections.

Placement of the intraoral coil spring involved a dental surgical procedure consisting of bur holes placed in the maxillary central incisors. The appliance was placed after being calibrated to deliver 15 g of laterally directed force. Half of the animals had either a passive appliance placed or no appliance at all.

The described grouping of animals made it possible to study the localization of BSA, endogenous rat albumin, tetracycline, and DCAF with and without the effect of an active intraoral coil spring.

At the end of the three day experimental period, the animals were sacrificed. Maxillary central incisors and surrounding bone and mid-palatal suture were dissected free

Experimental Condition	Route of Administration	# of Animals	Activated Intraoral Coil Spring # of Animals
Control (PBS)	IV	4	2
BSA	IV	4	2
Tetracycline	IP	4	2
Tetracycline	IM	4	2
DCAF	IP	4	2

TABLE I. Experimental design

under copious administration of 10% neutral buffered formalin. Tissue specimens were then placed in 10% neutral buffered formalin for 16 h in the cold room. One half of the specimens were decalcified in EDTA prior to embedding in order to evaluate the effect of demineralization on the efficiency of any bone tracer. Tissue segments were then trimmed and embedded in JB-4 (Polysciences, Inc., Warrington, Pennsylvania). Undecalcified specimens were sectioned at 1 μm with a diamond knife (LKB ultramicrotome, Type 8801A, Sweden) while decalcified tissues were sectioned at 7 μm utilizing steel knives (Spencer #820 microtome, American Optical Corp., Buffalo, New York).

All specimens were sectioned and stained with hematoxylin and eosin or toluidine blue for routine light microscopy. BSA was localized utilizing an indirect immunoperoxidase technique. Similar methods were used to visualize endogenous rat albumin in control sections. Tetracycline and DCAF sections were observed under ultraviolet light.

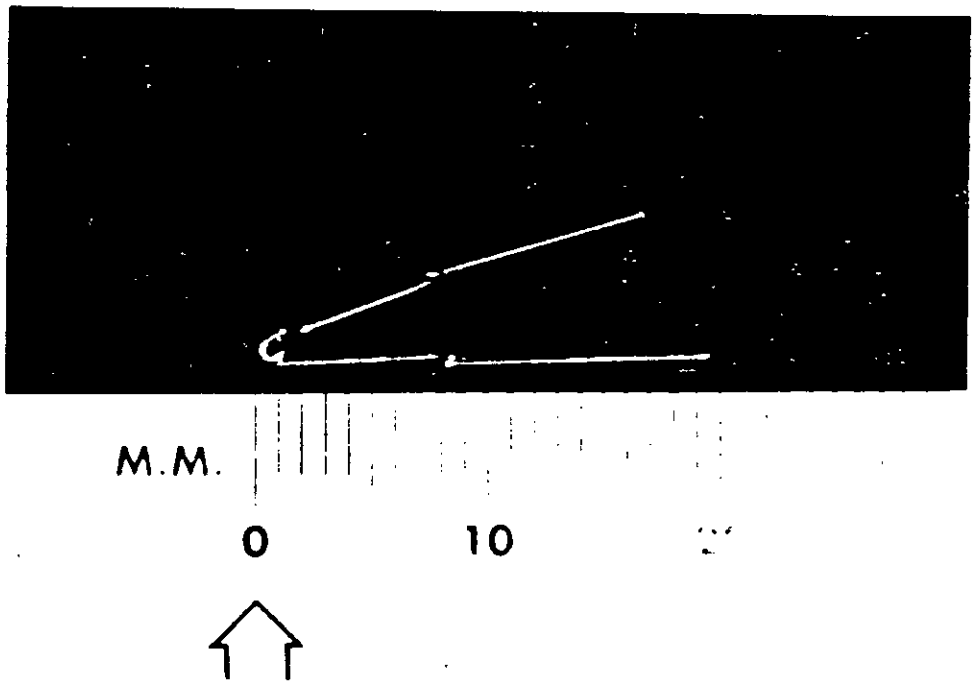
Appliance Construction

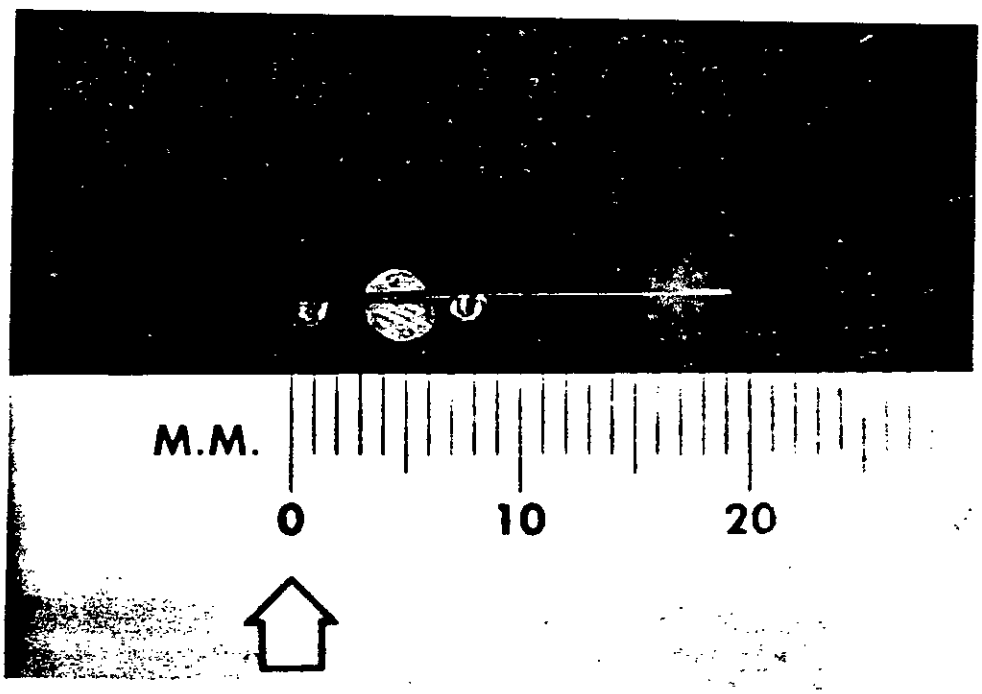
The appliance used to deliver the laterally directed force was first described by Storey (1955) and used by Stark and Sinclair (1987). It consisted of an intraoral coil spring constructed from 0.012 inch round austenitic stainless steel wire (Rocky Mountain Orthodontics, Denver, Colorado) (Plates 1, 2).

All springs were fabricated by first placing a single coil

Plate 1 - Top view of intraoral coil spring. Each Spring is calibrated to deliver a laterally directed force of 15 gms.

Plate 2 - Side view of spring. Extensions beyond the single coil are used to "thread" the appliance through the holes drilled in the rats' maxillary central incisors.


M.M.
0
10


M.M.
0
10
20

with a 5 mm extension in the wire. This was accomplished by wrapping the wire around the tip of a light wire plier (032-C) (Orthopli, Philadelphia, Pennsylvania). These extensions were used to "thread" the appliance through the holes drilled in the rats' maxillary central incisors. The single coil was used as a stop for spring placement.

The next step in the fabrication was to make two 9 mm arms separated by a multihelical coil. This coil was formed by placing three loops of wire around the tip of a light wire plier. The coil provided the flexibility in the spring to deliver a relatively constant force of 15 gms.

All springs were preadjusted with a precision force gauge (Haldex, Halmstad, Sweden) to produce 15 grams of force when activated. Placement of springs and final calibration is discussed in the surgical section.

Anesthesia

Initial induction of anesthesia for the appliance placement procedure and surgical intravenous injection technique was produced by inhalation of anesthesia grade ethyl ether (Fisher Scientific, Pittsburgh, Pennsylvania) in a closed bell jar until the animal was unconscious and breathing easily. This was followed by an intramuscular gluteal injection of 0.2 ml 90% ketamine hydrochloride solution (100 mg/ml Ketaset, Bristol Labs, Syracuse, New York) and 10% Acepromazine Maleate (10 mg/ml Ayerst Laboratories Inc., New York, New York). These drugs were

used in combination to facilitate handling of the animal and to increase the margin of safety over and above the use of a single drug. Ethyl ether inhalation rendered the animals unconscious and enabled precise injections for further anesthesia. The Ketaset was used to produce anesthesia which causes the animal to be immobile and free of pain. The Acepromazine was used as a tranquilizer which renders the animal relaxed.

Surgical Procedure

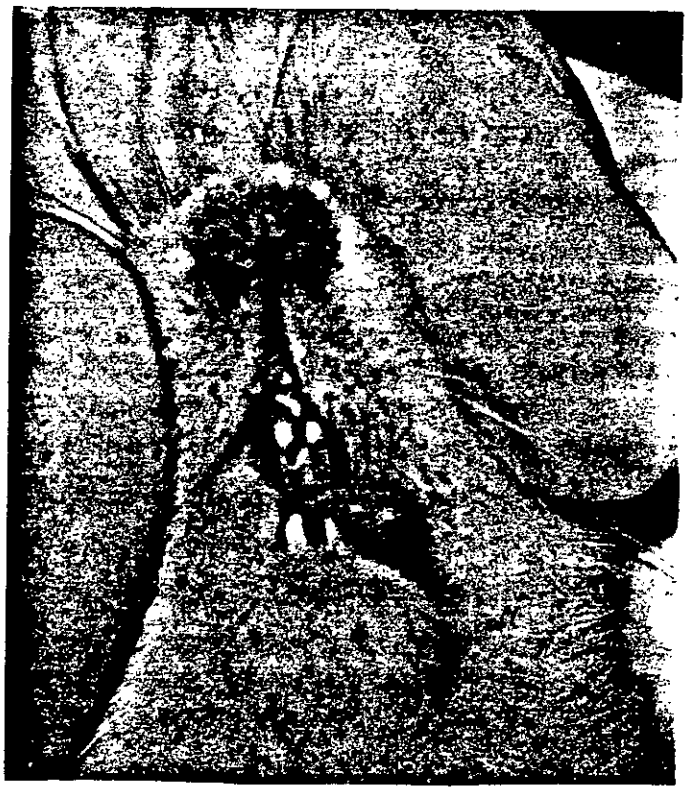
A. Appliance Placement

The purpose of this procedure was to provide a simple model for bone remodeling in response to orthodontic tooth movement and suture separation. This was accomplished by placement of an intraoral coil spring designed to direct lateral force to maxillary central incisors and across the premaxillary suture. Once the animals were anesthetized and weighed, a small hole was drilled from the labial to the lingual aspect in each central incisor with a ¼ round bur under copious water spray. The holes were placed approximately 2 mm from the incisal edge, thus avoiding the pulp while leaving adequate tooth structure for normal incisor wear occlusal to the holes (Plates 3, 4). After annealing the wire extensions, all springs were adjusted to 15 grams of force by inserting one wire extension of the spring into an incisor while the other wire extension was compressed to a position equivalent to the hole in the remaining incisor. Because all springs were preadjusted to deliver a force of 15

Plate 3 - Front-right view of rat oral cavity. A small hole is drilled from the labial to lingual aspect in each central incisor with a ¼ round bur.

Plate 4 - Holes completed





gms most were ready for placement without adjustment. The appliance was then placed intraorally along the palate of each animal with the annealed portions of the legs passing through the holes in the incisors. This portion was then bent back to lock the spring in place (Plate 5). Animals were now ready for appropriate injections.

B. Surgical Intravenous Injection Technique

A modified procedure utilizing a surgical technique was required to ensure a proper intravenous injection. BSA and PBS were administered in this manner. A pilot study utilizing tetracycline confirmed the effectiveness of the technique.

This surgery was performed after application of the intraoral coil spring. Animals were already anesthetized. Copious amounts of Povidone-iodine (Betadine, The Purdue Frederick Co., Yonkers, New York) were applied to the base of the tail with a cotton tip applicator and blotted dry with a sterile 2"x2" gauze. An incision approximately 10 mm long was made on the dorsal midline of the tail starting approximately 1 inch from the beginning of the tail. Lateral incisions were then made arcing approximately 90^{o} of the tail circumference toward the tail vein (Plate 6). The flap of tissue was then folded back to reveal the tail vein. The flap was further peeled back if necessary to enhance exposure of the tail vein. A proper intravenous injection utilizing a tuberculin syringe and 27 gauge needle was then performed (Plate 7).

Plate 5 - Intraoral coil spring locked in place

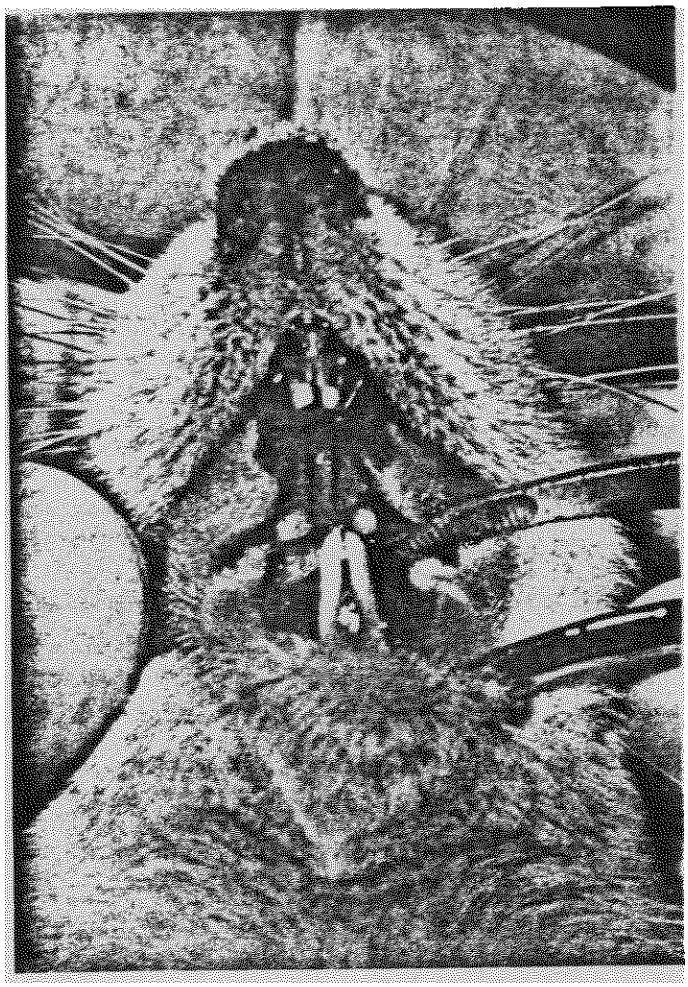
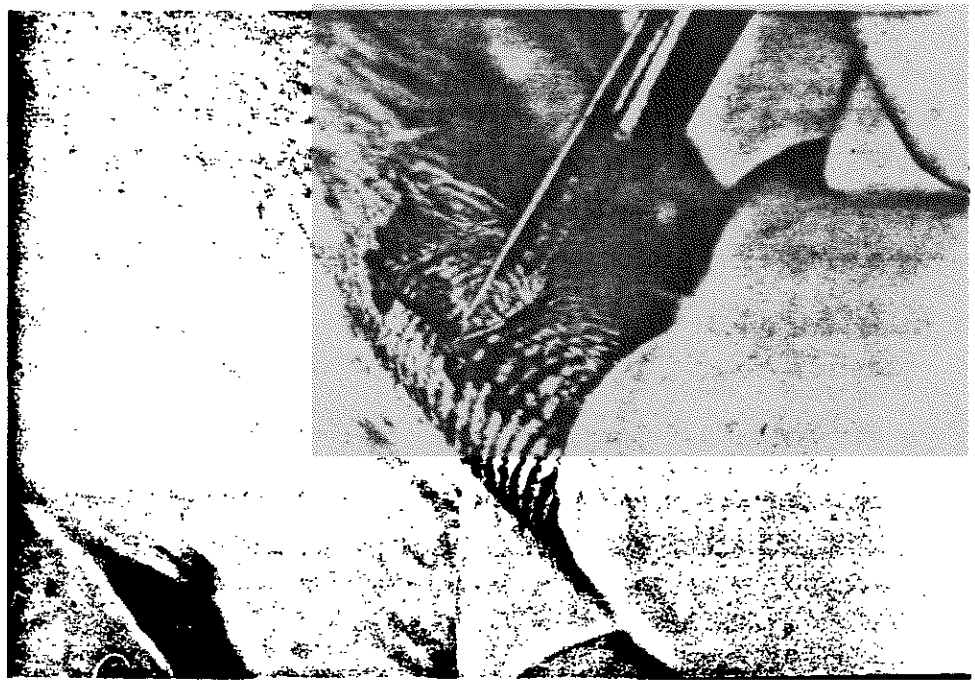
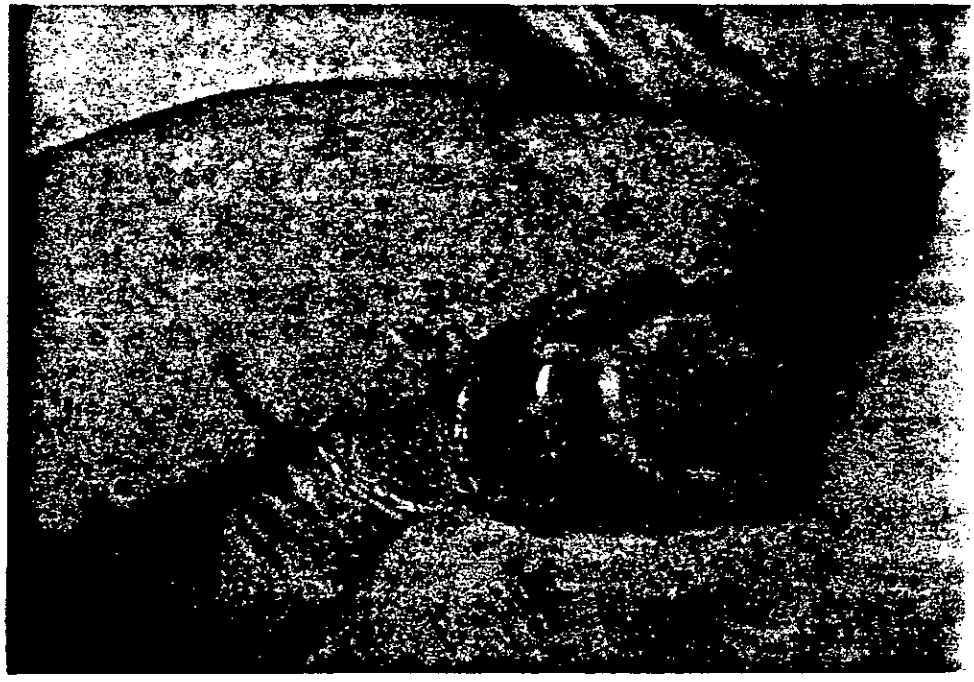


Plate 6 - Rat tail. Midline and lateral incisions are made to form a flap used to expose the tail vein.

Plate 7 - Flap is folded back to reveal tail vein. Proper intravenous injection is performed.





The surgical area was then lightly dusted with neomysin sulfate (Neosul II, Carson Chemical Inc., New Castle, Indiana) to prevent any post-operative infections. The incision was closed with 4-0 silk suture (Ethicon cutting CE-2, Henry Schein Co., Port Washington, New York) which was individually triple tied to prevent loosening.

The animals were transferred to a recovery cage. Once recovery was complete the animals were returned to their original cage.

Sacrifice of the animals

Sacrifice of the animals took place three days after the start of the experiment. After weighing, the animals were killed by an overdose of anesthesia grade ethyl ether by inhalation.

After death, a block section of the maxilla, including the incisor teeth and surrounding bone and the mid-palatal suture, was then surgically removed under copious administration of 10% neutral buffered formalin. Tissue specimens were then placed in 10% neutral buffered formalin for 16 h in the cold room and prepared for histologic processing.

Histologic Processing

Following fixation, tissue specimens were washed with PBS for 24 h in the cold room. At this point, one half of the specimens were decalcified in 0.5 M EDTA for seven days in the cold room. This was done in order to evaluate the effect of

demineralization on the efficiency of any bone tracer. The EDTA solution was changed at least once per day. After decalcification, specimens were again rinsed with PBS for 24 h in the cold room.

The specimens were then ready for infiltration and embedding in JB-4. Undecalcified sections were processed for infiltration and embedding immediately after the first PBS rinse. Decalcified sections were infiltrated and embedded after the second PBS rinse. All tissues were infiltrated by the following regimen: 70% ethanol, 45 min; 80% ethanol, 60 min; 90% ethanol, 60 min; equal amounts of absolute ethanol and JB-4 Solution A, 2 h. Each specimen was then placed in catalyzed Solution A using 0.1 g of benzoyl peroxide catalyst/10 ml Solution A for 32 h. Infiltration was carried out in the dark in the cold room with constant agitation on an orbital shaker.

Decalcified infiltrated tissue was embedded in BEEM capsules (Polysciences, Inc.) with a mixture of catalyzed Solution A and Solution B (1.0 ml/2.5 ml Solution A). The molds were placed in a bell jar under house vacuum as anaerobic conditions are needed for polymerization. Polymerization was complete at room temperature in two hours.

Undecalcified infiltrated tissue was embedded under a slightly different protocol. Catalyzed Solution A containing half the amount of benzoyl peroxide catalyst used for infiltrating the tissue was mixed with one half the recommended

amount of Solution B. The reduction in the proportion of solution to half the recommended amount produces a harder block which is necessary for successful sectioning of osseous tissue. Polymerization was complete at room temperature in 3 h.

Undecalcified specimens were sectioned at 1 μm on an ultra microtome with a diamond knife. This was done in an attempt to avoid the adverse effects on cellular preservation observed for ground or milled sections. Decalcified specimens were sectioned at 7 μm on a microtome utilizing steel knives. Sections were stained with hematoxylin and eosin or toluidine blue for routine descriptive histology. Additional sections were used for immunohistochemistry or observed under ultraviolet light.

Immunoperoxidase Localization

A. BSA

The method using peroxidase-labeled antibody for localization has been described in detail by Nakane and Pierce (1974) and Sternberger (1979).

1. Rehydration of Samples

JB-4 Embedded specimens from animals receiving BSA as well as controls were sectioned. Sections were floated on dH_2O, placed on glass slides, and incubated on a hot plate at 37°C overnite. Rubber O-rings were then glued to the glass slides to create an incubation well for the sections. A solution of 1% gelatin in PBS (GPBS) was pipetted into the wells and allowed to incubate for 2 h at room temperature to rehydrate the specimens.

2. Incubation with Anti-BSA Antibody

After fully hydrating, the 1% GPBS was poured off. A solution containing 1:50 diluted goat anti-BSA antibody (Sigma, St. Louis, Missouri) in 1% GPBS was then pipetted into the wells and allowed to incubate at room temperature for 2 h or overnight in the cold room.

3. Incubation with peroxidase conjugated rabbit anti-goat antibody

At the end of the first antibody incubation the specimens were washed with 1% GPBS to remove unbound antibody. Sections were then incubated with 1:100 diluted peroxidase conjugated rabbit anti-goat antibody (Sigma) in 1% GPBS for 2 h at room temperature. The specimens were then washed again and incubated in Diaminobenzidine (DAB) containing medium for 5 minutes. After thoroughly washing with 1% GPBS, the O-rings were removed and the sections were covered using Permount (Fisher Scientific). Sections were then ready for viewing under light microscope.

*DAB Containing Medium

To a solution containing 1 ml 0.5 M TRIS, pH 7.6 and 9 ml distilled H_2O (dH_2O) dissolve 5 mg DAB. Add 10 μl H_2O_2 just before use.

B. Endogenous Rat Albumin

Sections being stained for endogenous rat albumin by the indirect immunoperoxidase technique utilized the same protocol

as described previously for BSA. The only difference involved the first antibody incubation which was carried out using goat anti-rat albumin antibody (Sigma).

Enzyme-Linked Immunosorbant Assay

A. BSA

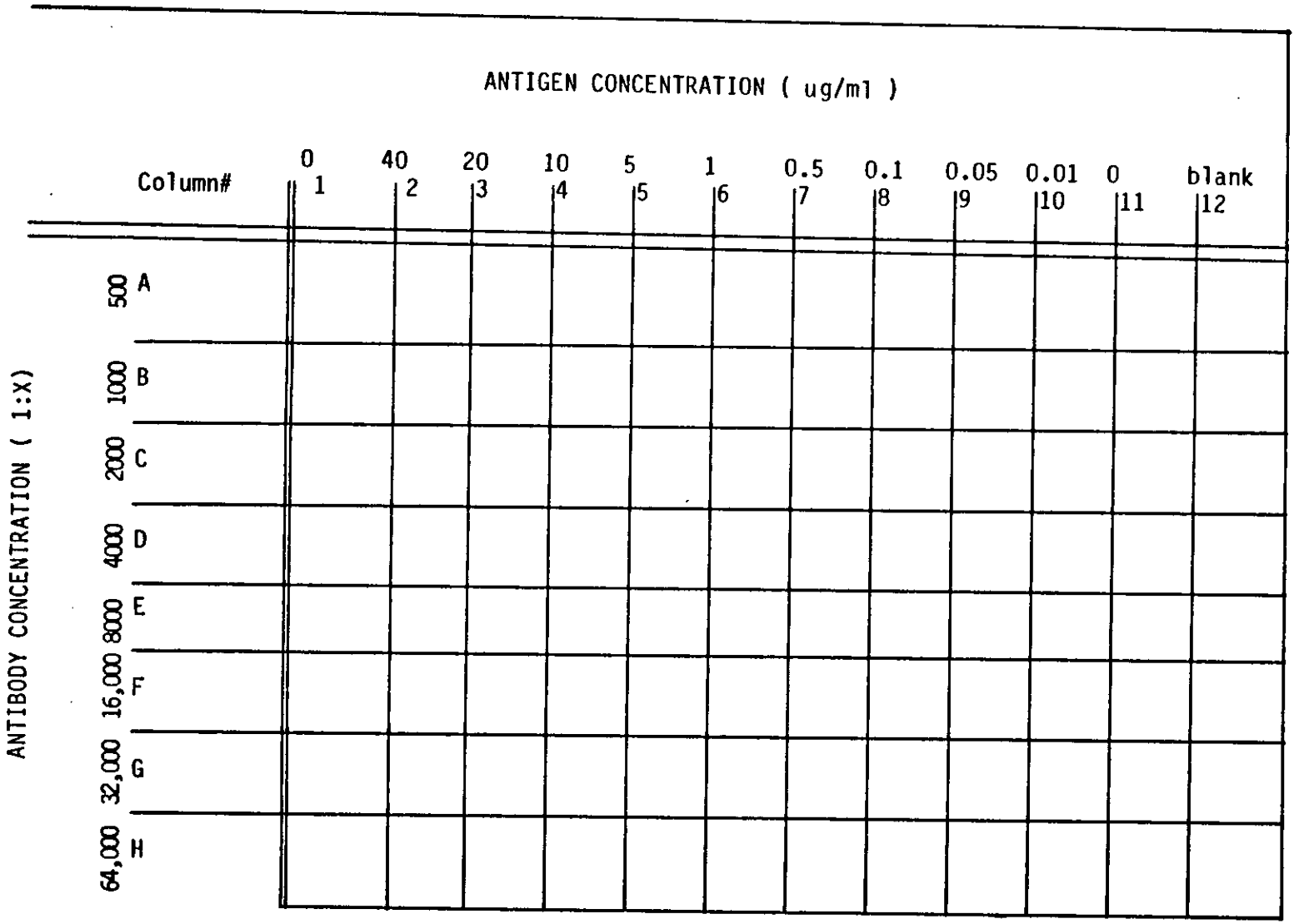
The affinity of commercially available antibodies was first assayed by ELISA. The indirect ELISA used in this study was essentially as described by Engvall and Perlmann (1971). The method employed an enzyme labeled second antibody with the antigen attached to a solid phase. The procedures are diagrammatically depicted in Figure 2.

1. Binding of BSA to Solid Support

96-well microtiter plates (Immulon I, Dynateck, Chantilly, Virginia) were used for the assay. These plates contained 12 wells in the horizontal direction (column nos. 1 to 12) and 8 wells in the vertical direction (row nos. A to H). Column no. 1 was used to zero the microplate reader (EL307C, Bio-Tek, Winooski, Vermont). No antigen was applied to these wells, but both antibodies were added in subsequent incubations. Column nos. 11 and 12 were used as controls. Column no. 11 received the second antibody and substrate while column no. 12 received substrate only.

BSA was first dissolved in coating buffer.* The antigen was then serially diluted. The concentrations of the diluted solutions ranged from 40 mg/ml to 10 ng/ml (Figure 2). 200 μl

Figure 2. Antigen concentration and antibody dilutions used in the ELISA and their arrangement in a microtiter plate.


ANTIGEN CONCENTRATION (ug/ml)
Column#
0
40
20
10
5
1
0.5
0.1
0.05
0.01
0
blank
1
2
3
4
5
6
7
8
9
10
11
12
ANTIBODY CONCENTRATION (1:X)
500 A
1000 B
2000 C
4000 D
8000 E
16,000 F
32,000 G
64,000 H

of each diluted antigen solution was applied to the wells in such a way that all wells in the same vertical direction had the same concentration of the antigen. The wells in column nos. 1 and 11 were filled with 200 µl of coating buffer without antigen. The solution was allowed to incubate in the wells for 2 h at room temperature (Figure 3A).

*Coating Buffer

To make 100 ml of coating buffer, 0.84g of $NaHCO_3$ was dissolved in dH_2O. The pH was adjusted to 9.0 prior to use.

2. Blocking Unbound Plastic Surface

After removal of the antigen solution, the area in the wells which was not covered by the antigen at the incubation step was now covered by gelatin. This was accomplished by filling the wells to the top with 1% gelatin in PBS* and allowing incubation for 1 h at room temperature

*1% gelatin in PBS (GPBS)

5g of gelatin, U.S.P. was dissolved in 500 ml of PBS, pH 7.4. 0.5 ml of Polyoxyethylene Sorbitan (Tween-20, Sigma) was then added to make a 0.1% solution.

3. Incubation with goat anti-BSA antibody

The affinity purified antibody was serially diluted with 1% GPBS in separate test tubes. The dilution of the antibody ranged from 1:500 to 1:64,000, with each dilution differing by a factor of 2. Two hundred µl of each properly diluted antibody was then applied to the microtiter plate in such a way that the

Figure 3. Schematic procedure of ELISA.

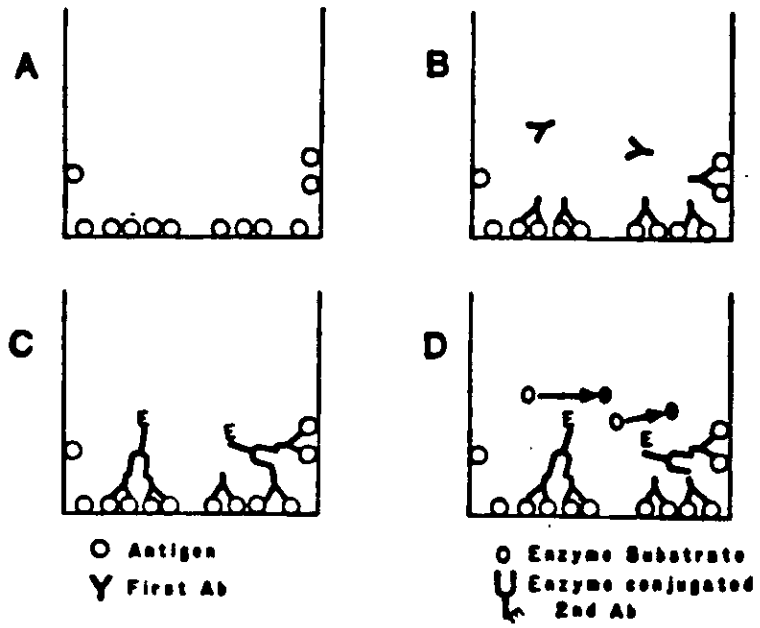
A. Antigen is absorbed to a microtiter well.

B. Specific antibody is added and binds to the immobilized antigen.

C. Enzyme conjugated second antibody is added and binds to the specific antibody.

D. Enzyme substrate is added and converted to product.

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A
B
C
D
E
E
E
E
O Antigen
Y First Ab
O Enzyme Substrate
Enzyme conjugated
2nd Ab

wells in the same horizontal direction had the same dilution of antibody (Figure 2). The incubation was allowed to continue for 2 h at room temperature or overnight in the cold room (Figure 3B).

4. Incubation with Peroxidase Conjugated Rabbit Anti-Goat Antibody

After removal of the anti-BSA antibody from the microtiter plate at the end of the incubation, the wells were washed three times with 1% GPBS. Rabbit anti-goat antibody was diluted 1:1000 with 1% GPBS and 200 μl of the solution was then added to the wells. Incubation time for this solution was 2 h at room temperature (Figure 3C).

5. Adding the Enzyme substrate and Reading the Result with the Microplate Reader.

The wells were washed three times with 1% GPBS after removal of rabbit anti-goat antibody. The substrate of peroxidase was prepared by adding 0.4g of O- phenylenediamine (OPD, Sigma) to 100 ml of the buffer made up of 0.73g of sodium phosphate monobasic and 0.51g of citric acid, pH 5.0. 30 μl of 30% H_2O_2 was added to the solution immediately before use.

200 μl of OPD solution was pipetted to all the wells in the microtiter plate including the wells in column no. 12. The length of incubation time was determined empirically. The color of the reaction product was judged visually until, in the darkest wells, the color was yellow brown. This usually took

about 5 min. The enzyme-substrate reaction was stopped by adding 40 µl of 2N HCl to each well (Figure 3D).

The reaction between peroxidase and the substrate converted colorless OPD solution into a chromophore. The resulting color was then measured in situ using the Bio-Tek microplate reader.

B. Endogenous Rat Albumin

ELISA performed to evaluate the specificity of antibody to endogenous rat albumin was performed as described for BSA. The antigen used was rat albumin prepared in concentration identical to those used for the BSA ELISA. In addition, the first antibody incubated in the microtiter plates was goat anti-rat albumin antibody. All other aspects of the procedure remained the same.

Inhibition ELISA

This method, as described by Rennard et al. (1982), was used in this study to determine the cross reactivity between rat albumin and BSA as a potential source of non-specific binding by goat anti-BSA antibody. Thus, rat albumin was used as the inhibitor.

To perform an inhibition ELISA, 200 µl of the BSA (20 ug/ml in coating buffer) was added to the microtiter plate and allowed to incubate for 2 h at room temperature (Figure 4A-1). In separate containers, goat anti-BSA antibody (1:1000 dilution) was mixed with various concentrations of inhibitors (Figure 4A-2). This was accomplished by adding small amounts of

Figure 4. Schematic procedure of an inhibition ELISA.

A1. Antigen is absorbed to the surface of the microtiter well.

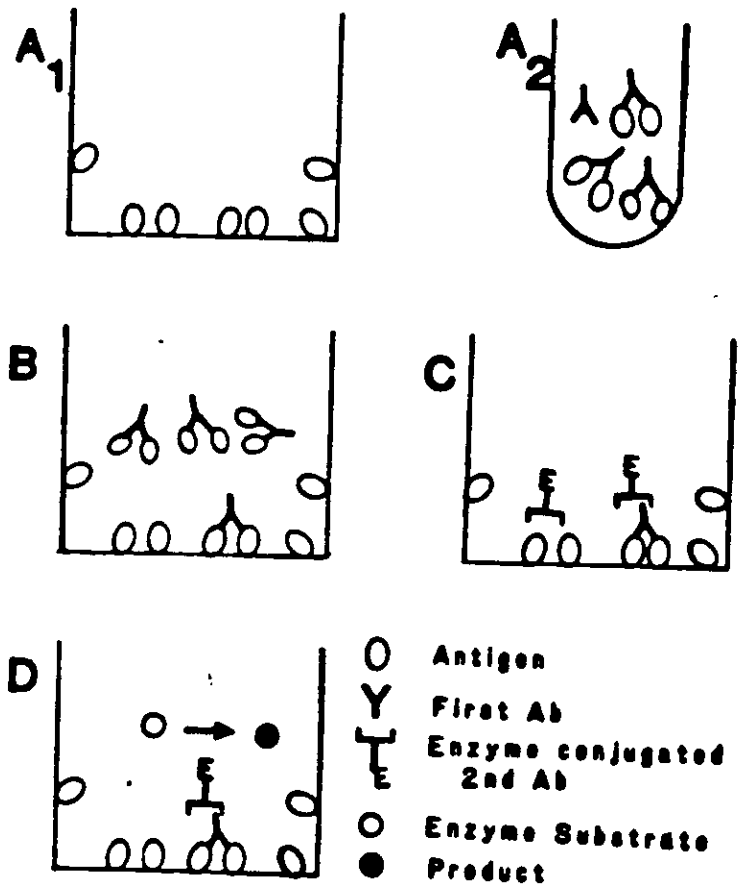
A2. Specific antibody is allowed to incubate with different amounts of inhibitors in a separate container.

B. Preincubated specific antibody is added and binds to the insoluble antigen.

C. Enzyme conjugated second antibody is added.

D. Enzyme substrate is added and converted to a color product.

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A1
A2
B
C
D
Antigen
First Ab
Enzyme conjugated 2nd Ab
Enzyme Substrate
Product

concentrated inhibitors (2 mg/100 µl in dH_2O) to 1:1000 diluted antibody solution and then serially diluting in the same solution. The final concentration of inhibitors ranged from 1 mg/ml to 0.1 µg/ml. The solution was incubated at room temperature for 2 h. This allowed the inhibitors to bind to the antibody, and when the mixture was subsequently transferred to the previously coated plastic wells, prior interaction of antibody with the inhibitors prevented some of the antibody from binding to the BSA absorbed onto the solid phase.

After coating the microtiter plate with BSA, the antigen free area in the wells was blocked by incubating with 1% GPBS for 1 h at room temperature. 200 µl of the antibody-inhibitor mixture were then pipetted to the wells and incubated at room temperature for 2 h or overnight in the cold room (Figure 4B). After removing the mixture, the wells were rinsed three times with 1% GPBS, leaving behind only the antibodies that bound to BSA. The subsequent steps of adding peroxidase conjugated rabbit anti-goat antibody and substrate (Figure 4C, 4D) were the same as those used in ELISA.

Visualization of Tetracycline and DCAF

Sections from animals administered tetracycline and DCAF as well as those injected with PBS for controls were observed using a Zeiss III RS photomicroscope (Carl Zeiss, Inc., Thornwood, New York) under ultraviolet light (Zeiss D-7082 epi-fluorescence condenser, exciter filter BP450-490, barrier filter LP 520).

Ultraviolet light is necessary to illuminate both tetracycline and DCAF bone markers. Both decalcified and undecalcified sections were examined to determine the effect of demineralization on the effectiveness of any bone marker.

RESULTS

Response of the Animals to the Experimental Procedure

All animals recovered from the surgical anesthesia rapidly. In approximately one hour they regained normal behavior and coordination. There appeared to be no differences among the groups upon visual inspection. The behavior of the experimental animals did not deviate from that of the controls. It must be noted, however, that all animals receiving an intraoral coil spring seemed to paw at the appliance upon first recovering from the procedures. This behavior was not observed beyond the first experimental day. The surgical incisions involved in the intravenous injection technique healed well without sign of inflammation or infection.

The weight gain of the animals was quite variable. It appears that the placement of an activated intraoral coil spring resulted in a weight gain in these animals approximately one half that of animals with a passive appliance (Table II).

Reaction to Intraoral Coil Spring

Visual inspection of the rats' oral cavity at the time of sacrifice revealed a significant separation of the maxillary central incisors in those animals receiving an activated intraoral coil spring (Plate 8). The separation ranged from 1.5 mm to 2 mm. No separation of incisors was observed for animals receiving a passive appliance.

Experimental Condition	Route of Administration	Intraoral Coil Spring	# of animals	Weight gain gms/day
Control (PBS)	IV	Active	2	4
		Passive	2	8
BSA	IV	Active	2	4
		Passive	2	9
Tetracycline	IP	Active	2	7
		Passive	2	15
Tetracycline	IM	Active	2	5
		Passive	2	9
DCAF	IP	Active	2	3
		Passive	2	8

TABLE II. Average daily weight gain

Plate 8. Separation of maxillary central incisors observed after placement of an activated intraoral coil spring.



The majority of appliances were well-tolerated and adverse tissue reactions were not observed. On one occasion, however, an incisor fractured leaving one end of the coil spring locked into place while the other was free floating. The particular animal involved had a passive appliance placed and the condition was not present until the third experimental day. In an additional case the multi-helical coil became embedded into the palatal gingiva causing a local inflammation. No difference was observed in the activity of these animals when compared to their counterparts.

Routine Histologic Results

Histologic evaluation utilized 7 μm decalcified sections and 1 μm undecalcified sections. All specimens were embedded in JB-4 prior to sectioning and stained with hematoxylin and eosin or toluidine blue. Transverse cross sections, perpendicular to the maxillary central incisors' long axis and including the surrounding bone and premaxillary suture, in addition to cross sections of the midpalatal suture were observed. Sections from animals with a passive appliance were characterized by a weak, haphazard deposition pattern of bone especially adjacent to the periodontal membrane. Separation of the premaxillary suture was largely reduced and new bone was less prominant adjacent to the suture. Less osteoid was present and some osteoclastic activity and Howship's lacunae were observed. Larger, more numerous areas of broadly dispersed, loose connective tissue were

observed. Minimal numbers of osteogenic cells were seen lining bone surfaces.

In contrast, sections of the premaxillary suture from animals receiving an active intraoral coil spring were characterized by greater separation of the suture and increased deposition of bone adjacent to the premaxillary suture. Strong, consistant reversal lines delineating old and new bone were regularly observed. New bone appeared in a somewhat orderly pattern especially adjacent to the suture. Large amounts of osteoid were present and the area was characterized by immature bone and young osteocytes. (Plates 9-14)

Cross sections of the mid-palatal suture revealed an extremely variable and often complex suture. No discernable differences were noted between sections taken from animals with an active appliance versus those with a passive appliance. Relatively smooth bone surfaces were joined by a dense connective tissue containing many basopholic cells. The suture often took an extremely irregular course resembling a labyrinth while at other times appeared to be a simple butt joint. Some sections contained remnants of the nasal septum. (Plates 15-18)

Demineralized sections demonstrated good cell and tissue preservation. Large sections of varying tissue types were available for observation. Undemineralized sections were necessarily smaller in scope. However, cellular and tissue preservation was fairly good and regular section thickness of 1

Plate 9 - Decalcified premaxillary suture; H and E, 100x. Incisors, periodontal membrane, suture, and supporting bone all visible and intact. Sharpey's fibers can be seen inserting in bundle bone. Sparse collections of osteogenic cells line what is mostly compact bone.

Plate 10 - Undecalcified premaxillary suture; toluidine blue, 160x. Smaller block face in undecalcified specimens did not permit inclusion of junction between periodontal membrane and the teeth. However, ultramicrotome sectioning with a diamond knife allowed thin (1 μm) sections with good cellular preservation not obtainable with ground sectioning techniques. Compact bone of the suture is lined by some osteogenic cells and minimal osteoid.





Plate 11 - Unstressed premaxillary suture; toluidine blue, 160x. Smoother bone surfaces are present with less osteoid. Evidence of osteoclastic action is evident in Howship's lacunae.

Plate 12 - Unstressed premaxillary suture, higher magnification; 400x toluidine blue. Minimal remodeling changes are apparent. Good cellular preservation of young osteocytes.





Plate 13 - Stressed premaxillary suture; toluidine blue, 160x. Characterized by greater suture separation and bone deposition. Numerous osteoblasts, young osteocytes, osteoid, immature bone, and reversal lines are visible.

Plate 14 - Stressed premaxillary suture, higher magnification; 400x toluidine blue. Significant bone deposition is observed on the suture side of bone.





Plate 15 - Midpalatal suture; toluidine blue, 100x. Extremely serrated suture unsuitable as model for stress induced remodeling. Suture comprised of highly cellular connective tissue with osteogenic cells on smooth bone surfaces.

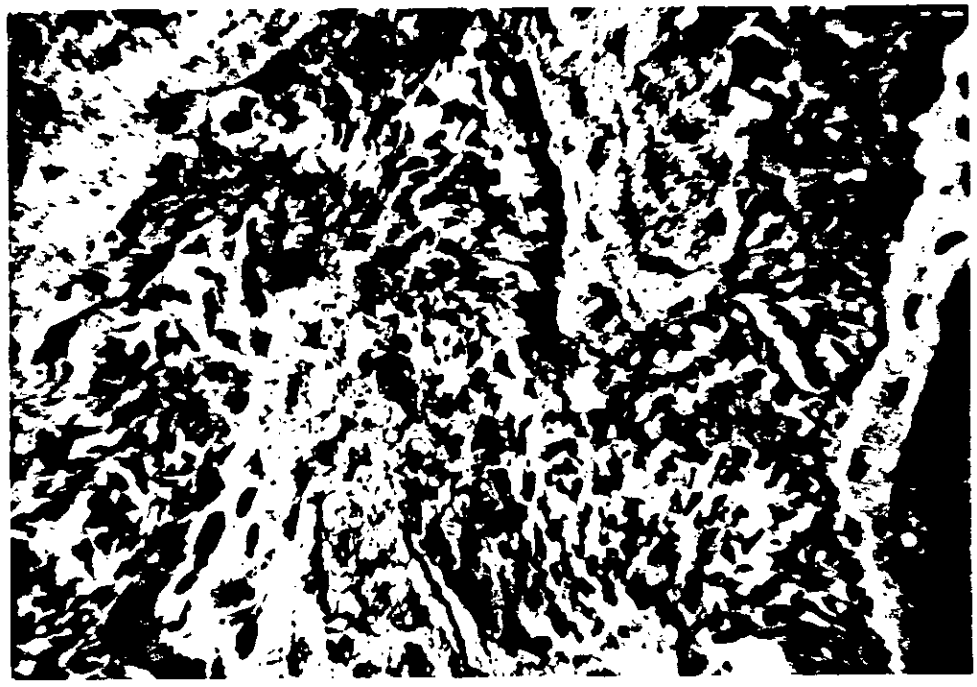
Plate 16 - Midpalatal suture; toluidine blue, 100x. Demonstrating variability of the suture. Remnants of nasal septum are visible.





Plate 17 - Unstressed midpalatal suture; toluidine blue, 400x. Smooth bone surfaces with cellular fibrous connective tissue.

Plate 18 - Stressed midpalatal suture; toluidine blue, 400x. No differences noted.





μm were routinely obtained.

There was no evidence of an inflammatory reaction or necrosis in any section. All bone and sutural tissues as well as periodontal membrane appeared non-pathologic and viable.

Immunoperoxidase Localization

A. BSA

Sections of the premaxillary suture incubated with affinity purified anti-BSA antibodies, followed by the peroxidase conjugated antibody sequence, showed a consistent pattern of light brown staining in the suture, and adjacent periodontal membrane. Osteocytes in the bone adjacent to the suture also stained light brown. These observations were made for decalcified as well as undecalcified specimens. In addition, decalcified sections were characterized by light brown staining in the dentin of the incisors. The pulps of the incisors showed a stronger staining pattern including fibroblasts and capillary endothelial cells. Red blood cells stained a dark redish brown. The application of an activated intraoral coil spring apparently had no effect on the staining intensity although greater suture separation was observed (Plate 19).

Although the antibody specificity was tested by means of ELISA, control sections were also exposed to anti-BSA antibody followed by the immunoperoxidase staining sequence in order to help determine to what degree the amount of staining seen in BSA sections was due to non-specific background. All in all,

similar staining patterns were seen in control sections as compared to BSA sections and no distinguishable differences could be noticed (Plates 20).

From the results of the control experiments, it was evident that the immunostaining of the premaxillary suture was non-specific for BSA.

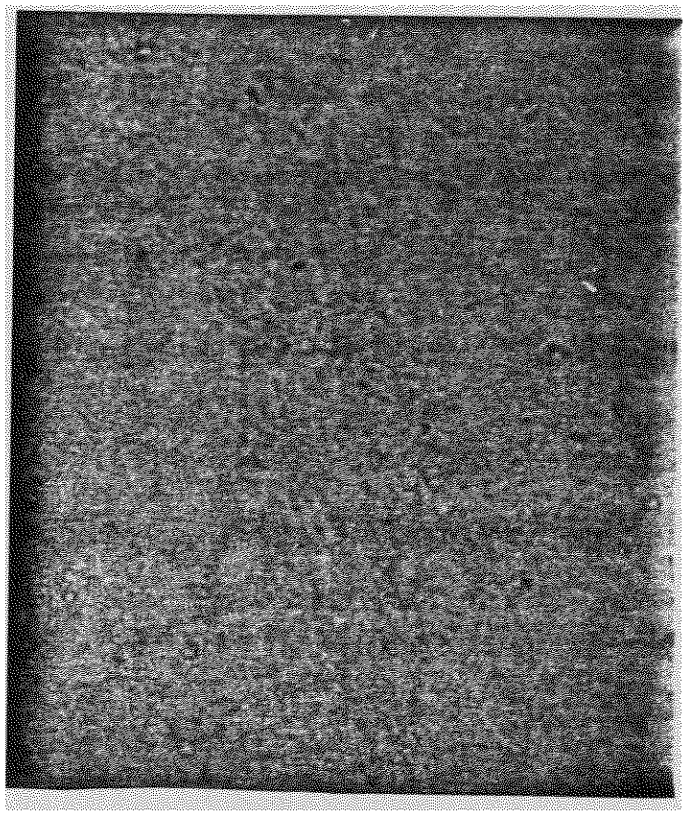
Sections of the palatal suture were also immunostained by the same procedures because it was thought that the force administered across the premaxillary suture would have a distant effect at the mid palatal suture. Palatal suture sections from animals receiving injections of BSA showed a light brown staining pattern within the suture and osteocytes in surrounding bone. Capillary endothelial cells and red blood cells stained a deep redish borwn. No distinction could be made based on the application of an active appliance (Plates 21). Control sections stained in a similar fashion (Plates 22). Again, it was evident that any staining seen in BSA sections could be attributed to non-specific background.

B. Endogenous Rat Albumin

Tissue specimens were immunostained for endogenous rat albumin by methods similar to that used for localization of BSA. This was done to confirm the presence of endogenous albumin within the calcified matrix. Both the premaxillary suture region and midpalatal suture stained ubiquitously regardless of whether the specimens were decalcified or not. The sutures and

Plate 19 - Immunostained premaxillary suture; BSA, 250x.
Light brown staining of the suture and osteocytes.

Plate 20 - Immunostained premaxillary suture; control, 250x.
No differences noted. Therefore, staining observed is due to non-specific background.



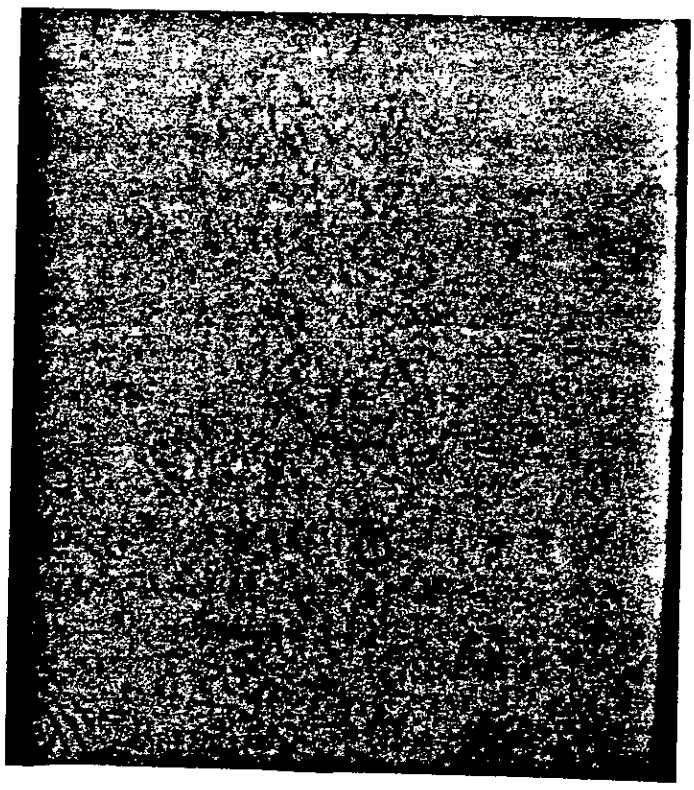
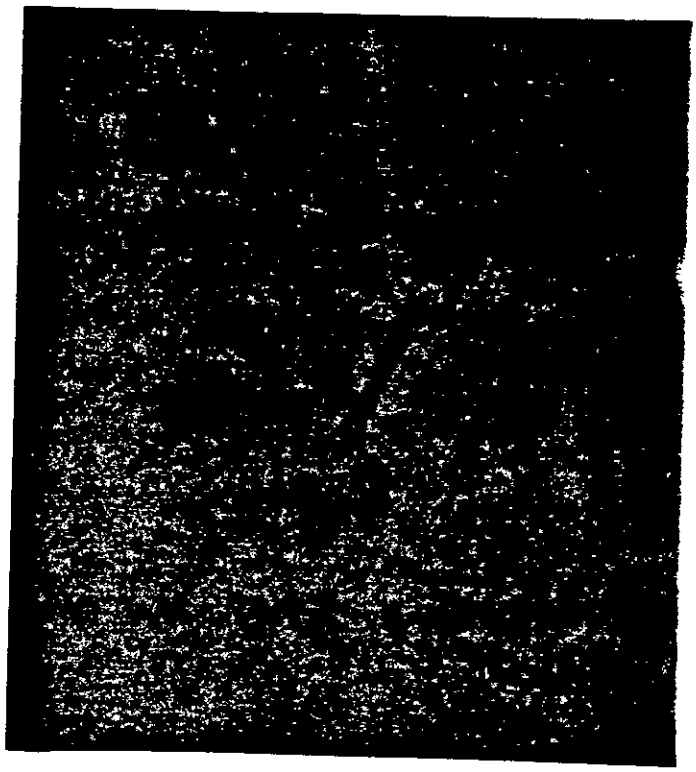
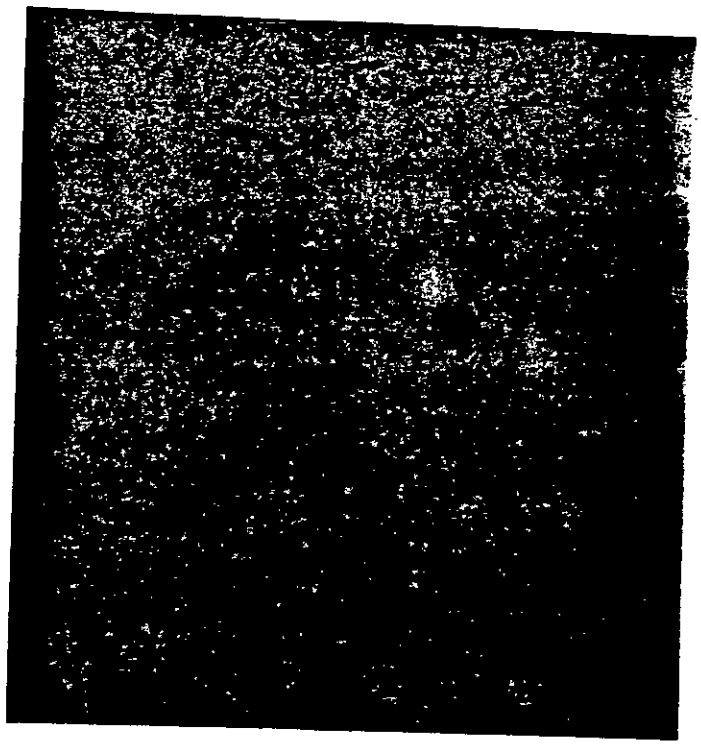


Plate 21 - Immunostained midpalatal suture; BSA, 160x. Light brown staining of the suture and osteocytes.

Plate 22 - Immunostained midpalatal suture; control, 160x. No differences noted; therefore, non-specific.





periodontal membrane stained slightly darker than bone. Osteocytes also stained slightly darker. Capillary endothelial cells and red blood cells stained a deep redish brown. Other than a greater separation of the premaxillary suture, no effects were observed due to placement of an activated intraoral coil spring (Plates 23, 24).

ELISA

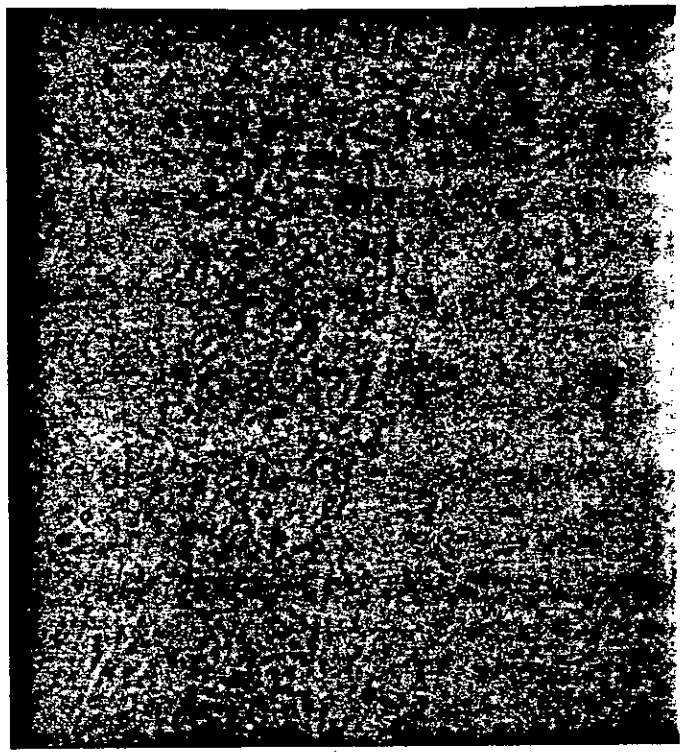
1. BSA

Enzyme immunoassay was employed to determine the specificity of the affinity purified antibody for BSA. The indirect ELISA was used because of its simplicity and sensitivity. The method does not require the conjugation of an enzyme to the specific antibody, which usually results in a decrease of the binding ability of the antibodies. On the other hand, the use of enzyme-conjugated second antibody increases the sensitivity of the test because two or more second antibodies can bind to one first antibody molecule, accordingly amplifying the result several fold.

The ELISA using BSA and affinity purified antibodies is shown in Figure 5. The products of the peroxidase reaction were measured at 405 nm and plotted against the concentration of BSA used to coat the plate. A family of curves was generated when different dilutions of antibody were used. A decrease in BSA concentration resulted in a smaller amount of enzyme bound and less color produced. However, results from antibody dilution

Plate 23 - Immunostained premaxillary suture; rat albumin, 250x. The suture, bone, and periodontal membrane are immunostained ubiquitously.

Plate 24 - Immunostained midpalatal suture; rat albumin, 160x. The suture and bone stain ubiquitously.



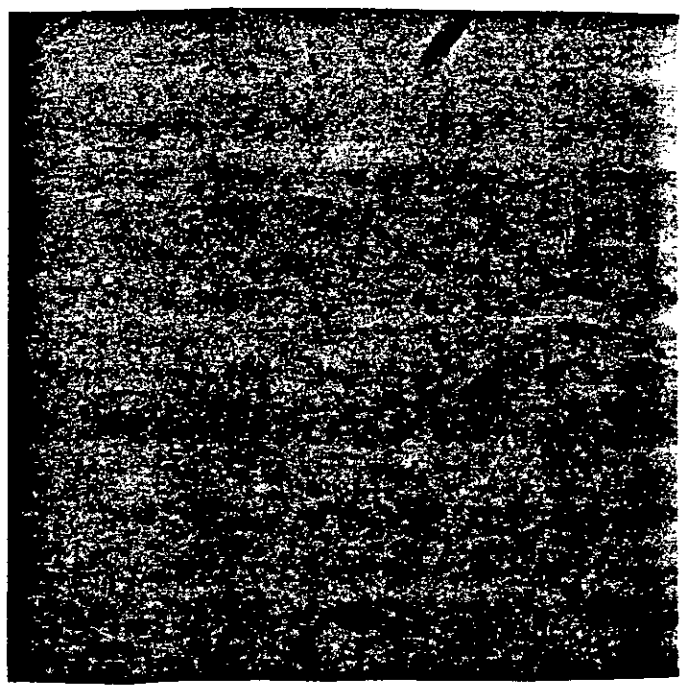
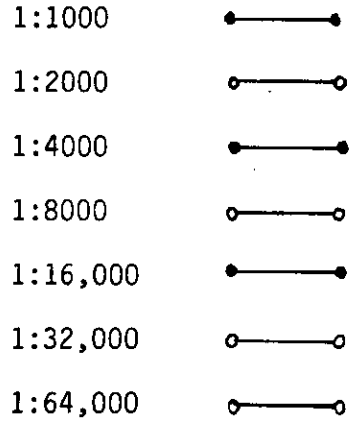
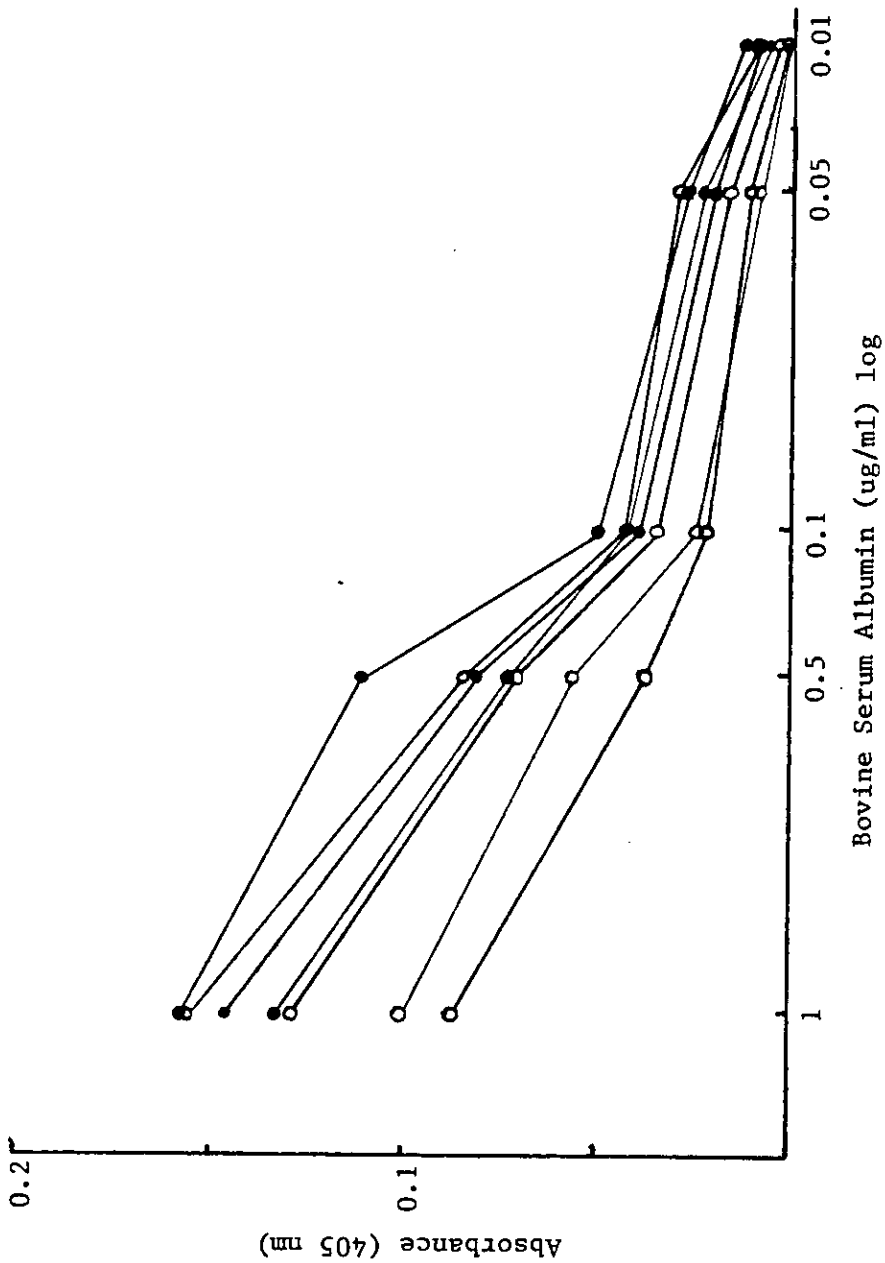


Figure 5 - ELISA (BSA).

The product of the enzyme reaction was measured at 405 nm and plotted against the concentration of BSA. A family of curves was generated when different dilutions of antibody were used.




Absorbance (405 nm)
0.2
0.1
Bovine Serum Albumin (ug/ml) log
1
0.5
0.1
0.05
0.01

were more variable and did not necessarily result in less color intensity.

2. Endogenous Rat Albumin

ELISA was also used to ascertain the specificity of affinity purified antibodies for endogenous rat albumin. Methods used were identical to that used for BSA. Endogenous rat albumin was used as antigen and goat anti-rat albumin was the first antibody incubated.

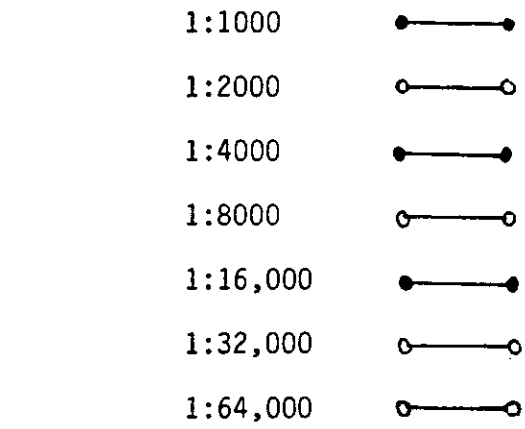
Results are shown in Figure 6. A decrease in rat albumin concentration resulted in a decreased amount of enzyme bound and less color produced. However, antibody dilution produced variable results. Increased antibody dilution did not necessarily result in less color intensity.

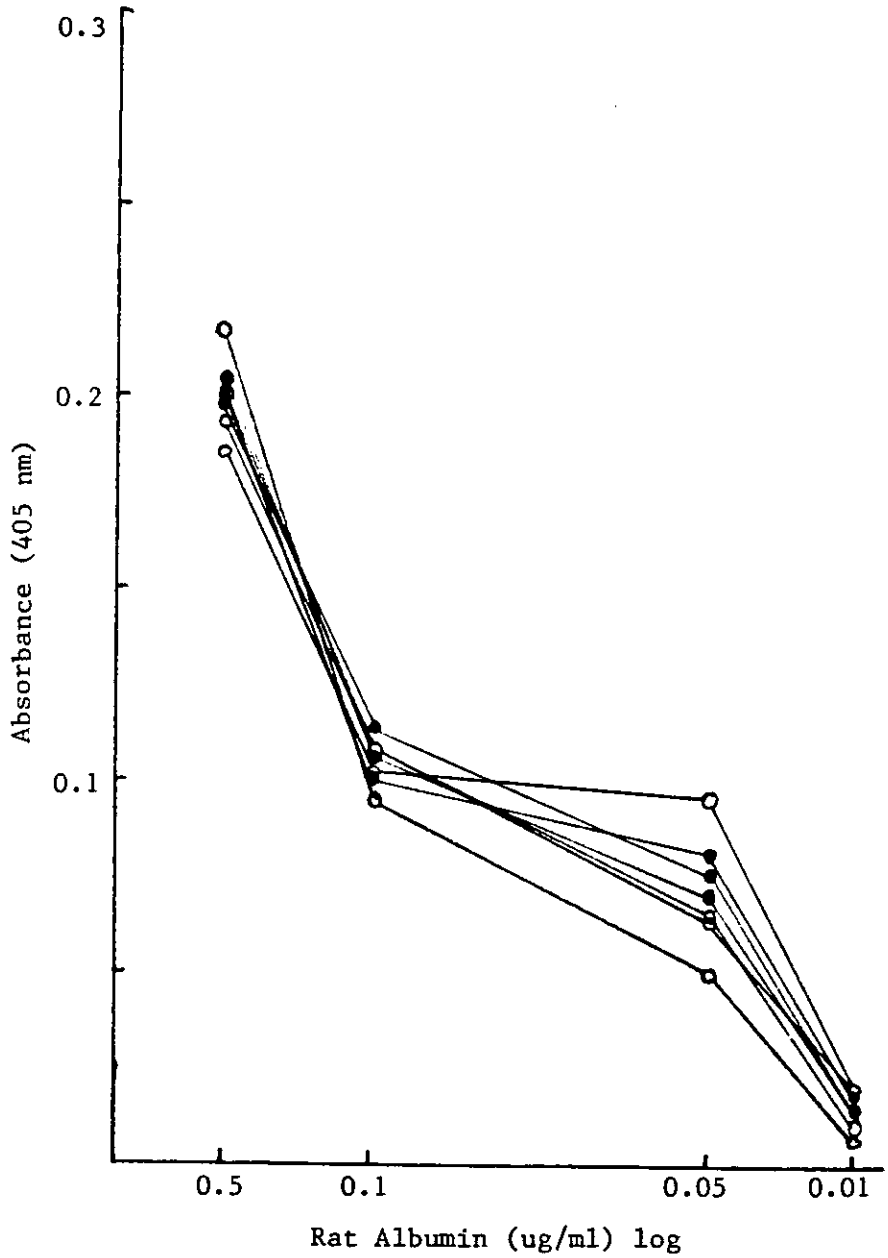
Inhibition ELISA

The inhibition ELISA procedure was used to check the cross reactivity between rat and bovine albumin. The principle of the inhibition ELISA is based upon the ability of the tested inhibitors to bind to specific antibodies and block the binding of these antibodies to the adsorbed antigen. The degree of inhibition depends on the structural similarity between the antigen and inhibitor.

An inhibition relationship was determined by using various amounts of endogenous rat albumin to inhibit the reaction between 20 μg/ml of BSA and 1:100 diluted specific antibody. The result is plotted as percent inhibition versus concentration

Figure 6 - ELISA (Rat Albumin)




0.3
0.2
0.1
Absorbance (405 nm)
0.5
0.1
0.05
0.01
Rat Albumin (ug/ml) log

of the inhibitor (Figure 7).

A standard inhibition curve could not be obtained. While inhibition was observed, the amount was not related to the concentration of endogenous rat albumin used as an inhibitor. Thus, the results indicate that cross reactivity between rat and bovine albumin cannot be confirmed, however, nor can it be ruled out.

Visualization of Tetracycline and DCAF

Sections taken from animals injected with tetracycline were observed under ultraviolet light to illuminate the tetracycline bone markers. This was done to create a baseline of information for the evaluation of BSA as a potential bone tracer. Tetracycline was administered both intramuscularly and intraperitoneally. Both methods appeared to be effective. No significant differences were noted (Plates 25, 26).

Undecalcified sections of the premaxillary suture showed increased amounts of diffusely labeled newly formed woven bone and an increased thickness of uncalcified matrix and amorphous ground substance. This was especially true for specimens from animals receiving an activated intraoral coil spring. In addition, the activated appliances caused an increase in the width of the suture while bone directly adjacent to the suture had a wider band of tetracycline incorporation when compared to bone adjacent to the periodontal membrane. (Plates 27-30)

Undecalcified sections of the midpalatal suture also showed

Figure 7 - Inhibition of the binding of anti-BSA antibody to microtiter plate coated with BSA by endogenous rat albumin.

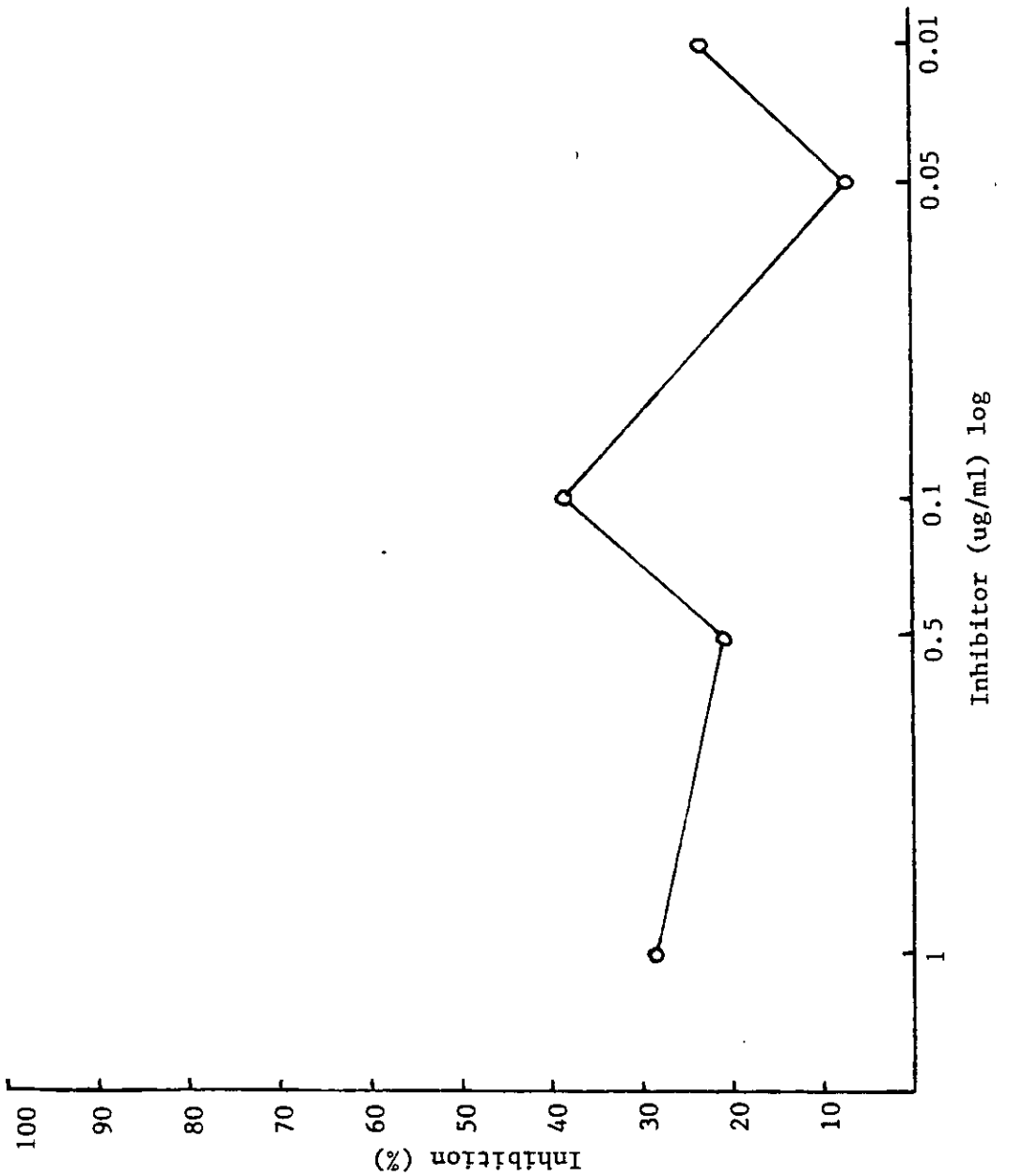
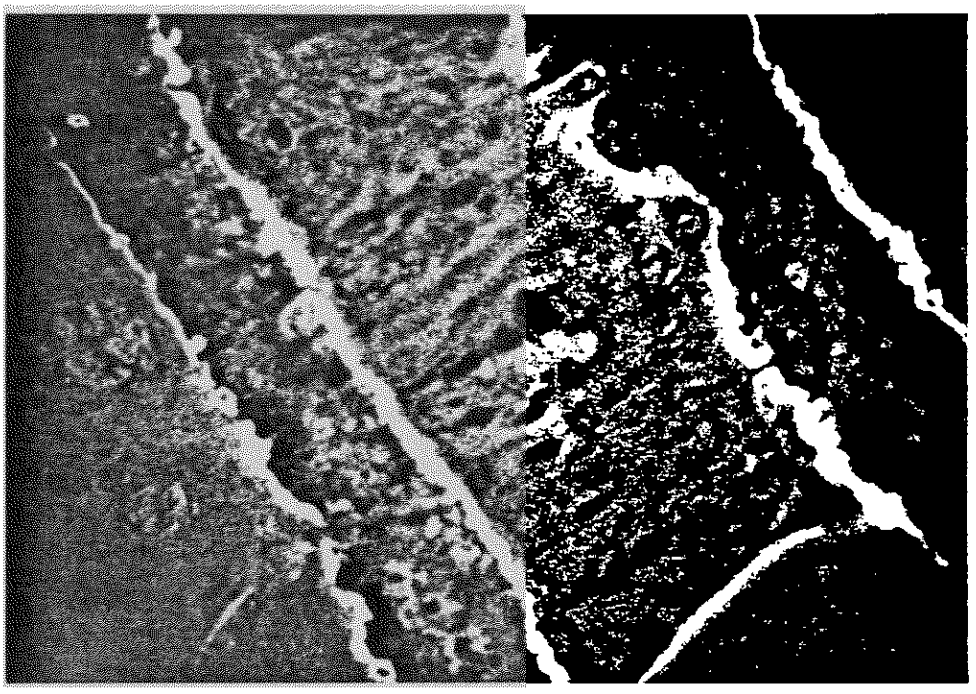

Inhibition (%)
100
90
80
70
60
50
40
30
20
10
1
0.5
0.1
0.05
0.01
Inhibitor (ug/ml) log

Plate 25 - Premaxillary suture; tetracycline IP, 160x. Fluorescence is observed on all bone surfaces.

Plate 26 - Premaxillary suture; tetracycline IM, 160x. Similar fluorescence is observed.



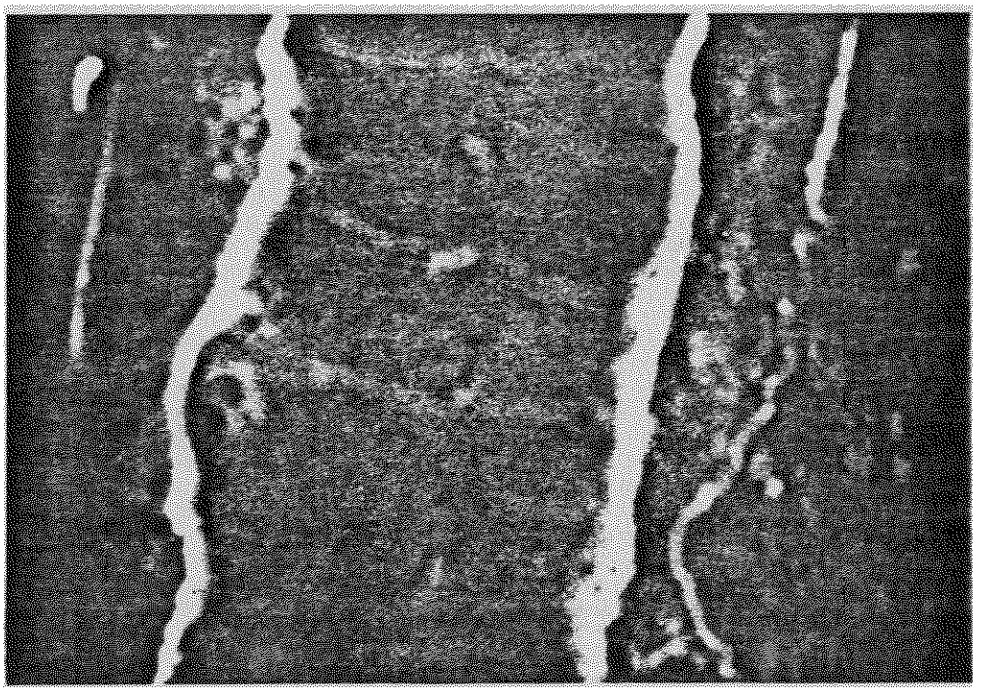
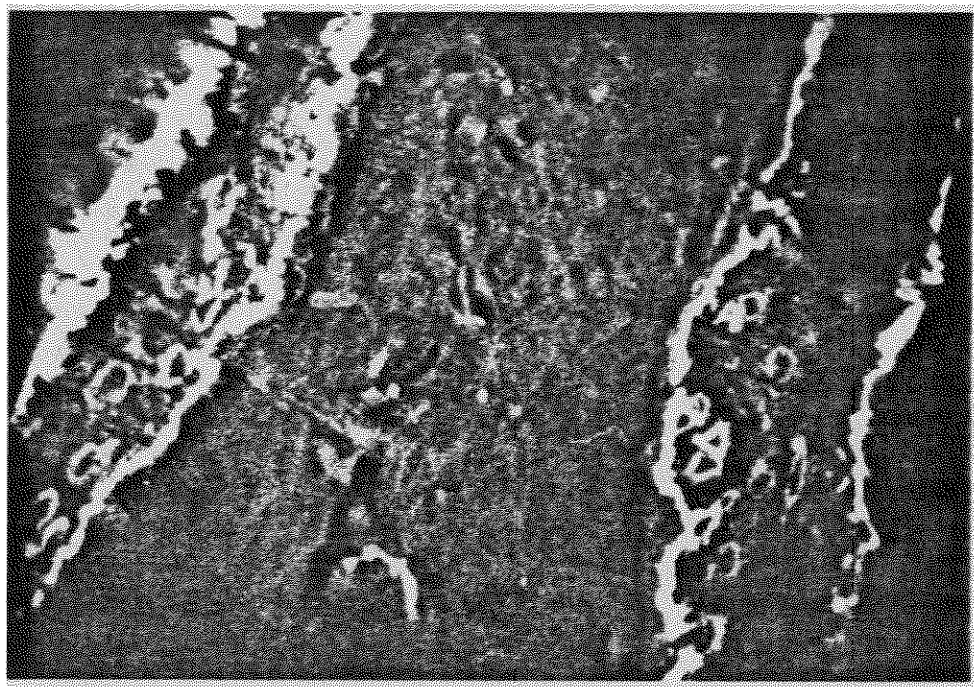


Plate 27 - Unstressed premaxillary suture; tetracycline, 250x. Equal fluorescence is seen on all bone surfaces.

Plate 28 - Unstressed premaxillary suture, higher magnification; 400x. tetracycline. Equal fluorescent banding.



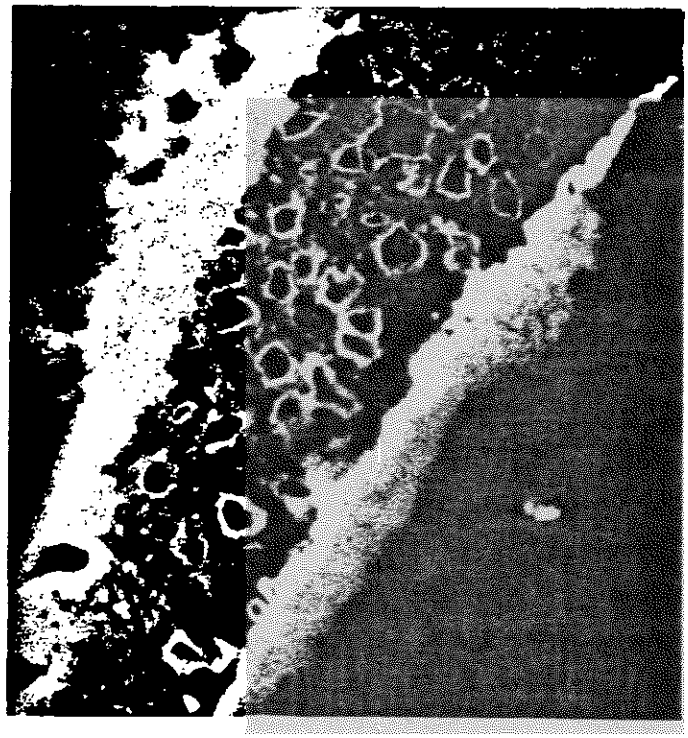
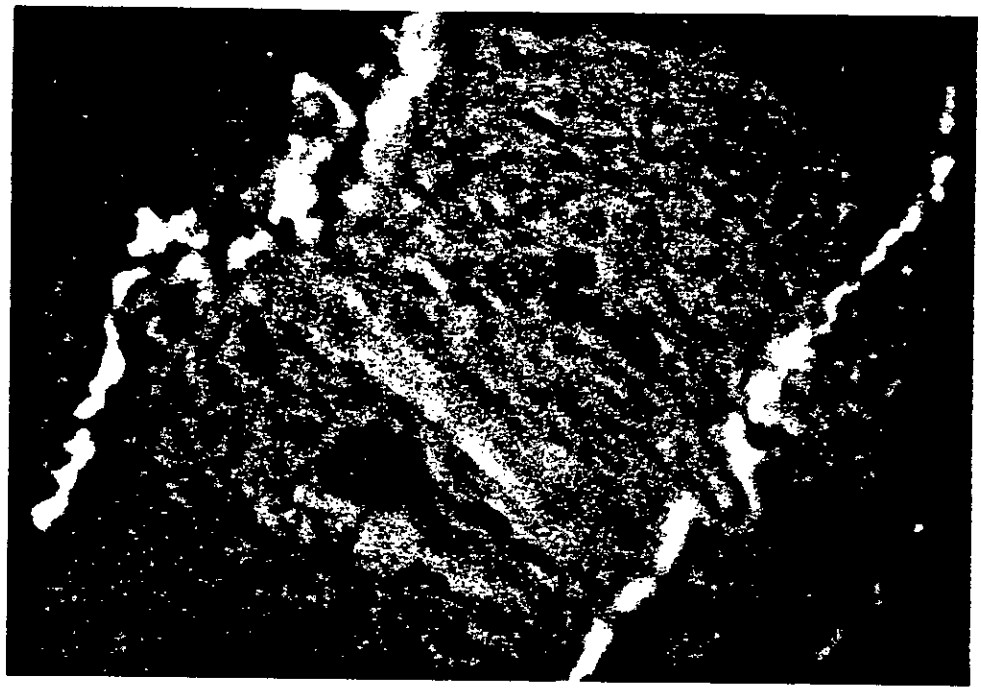
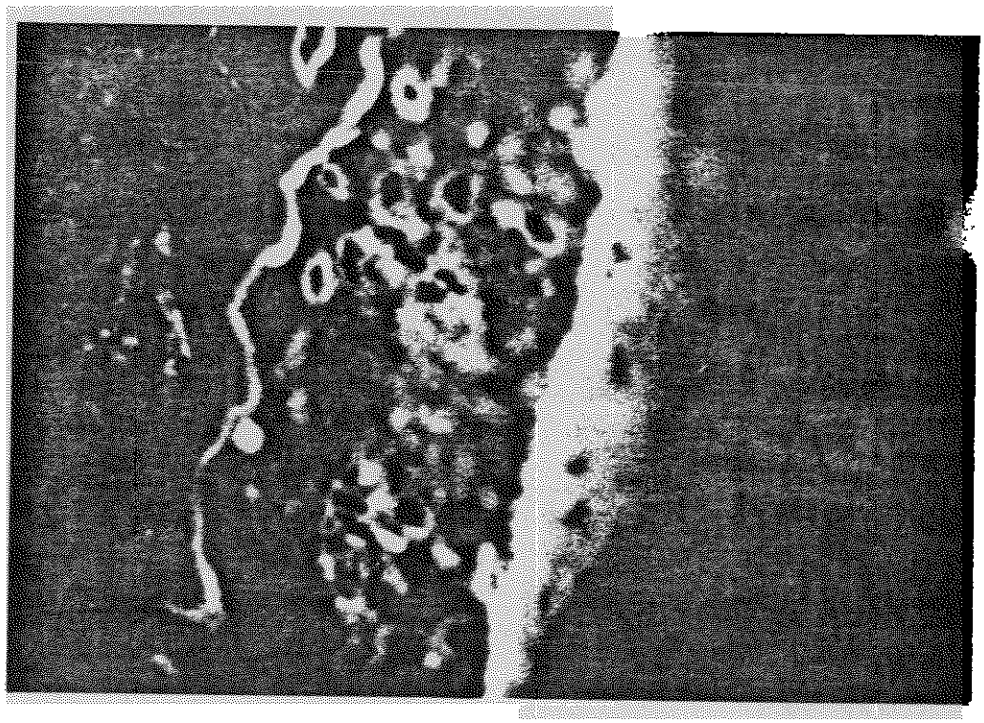


Plate 29 - Stressed premaxillary suture; tetracycline, 250x. Greater suture separation. Stronger fluorescence is seen adjacent to the suture. Minimal fluorescence is seen adjacent to the periodontal membrane.

Plate 30 - Stressed premaxillary suture, higher magnification; 400x. tetracycline. Thick fluorescent band adjacent to the suture. Bone deposition is readily observed.





characteristic tetracycline fluorescence under ultraviolet light. However, no difference was observed between groups receiving an activated appliance and those receiving a passive appliance. (Plates 31, 32)

Control sections were also observed in order to rule out autofluorescence and provide a comparison for tetracycline-and DCAF-injected specimens. Again, all sections were devoid of fluorescence regardless of tissue type or placement of an activated appliance (Plates 33, 34).

Decalcified sections were observed to determine the effect of demineralization on the efficiency of any bone tracer. The effect of demineralization was quite dramatic. As a result, fluorescence was no longer detectable in any region regardless of tissue specimen or the placement of an activated coil spring. (Plates 33, 35)

Sections taken from animals receiving injections of DCAF were also observed under ultraviolet light. Thus, additional qualitative information was obtained to evaluate BSA as a potential bone tracer.

Findings for DCAF sections were similar to tetracycline results (Plates 36, 37). DCAF fluorescence appeared to be slightly more distinct than tetracycline. In addition, the difference between osteoid and newly calcified matrix was more clearly defined. All other findings were coincident (Plates 38-43). Decalcified sections were devoid of fluorescence (Plates 44-46).

Plate 31 - Unstressed midpalatal suture; tetracycline, 250x.
Fluorescence is seen on most bone surfaces.

Plate 32 - Stressed midpalatal suture; tetracycline, 400x.
Similar fluorescence observed.

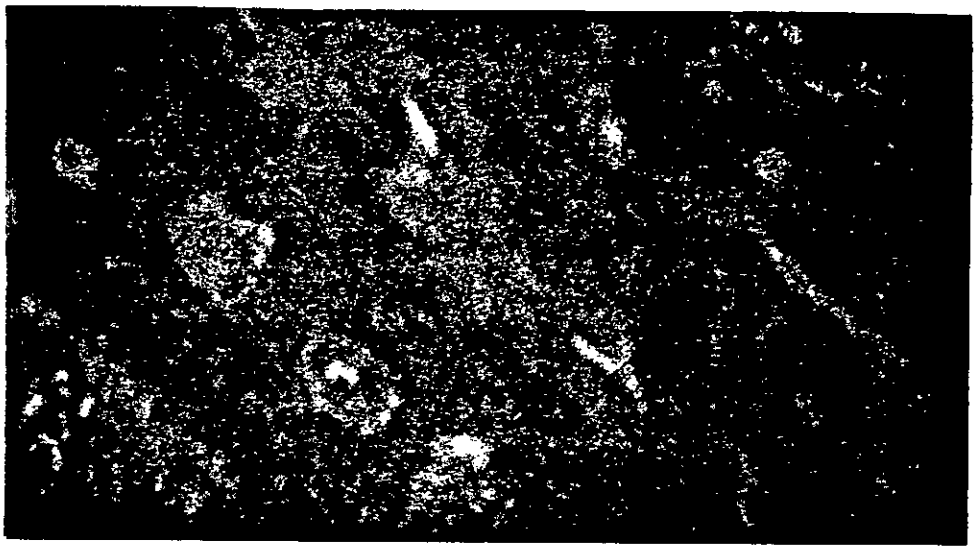


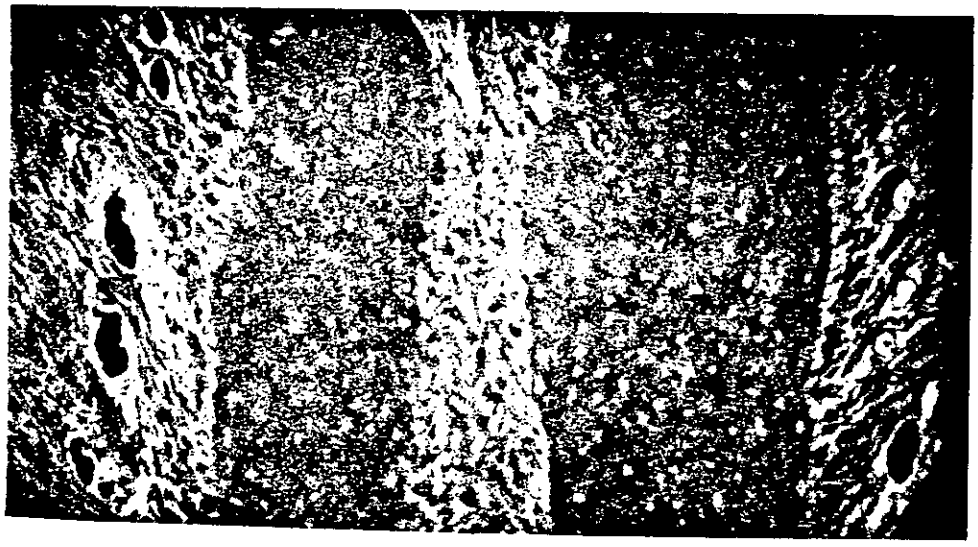


Plate 33 - Undecalcified premaxillary suture; tetracycline, 160x. Routine fluorescence observed.

Plate 34 - Undecalcified premaxillary suture; UV control, 160x. Minimal auto fluorescence.

Plate 35 - Decalcified premaxillary suture; tetracycline, 160x. Minimal fluorescence. Tetracycline completely washed out.





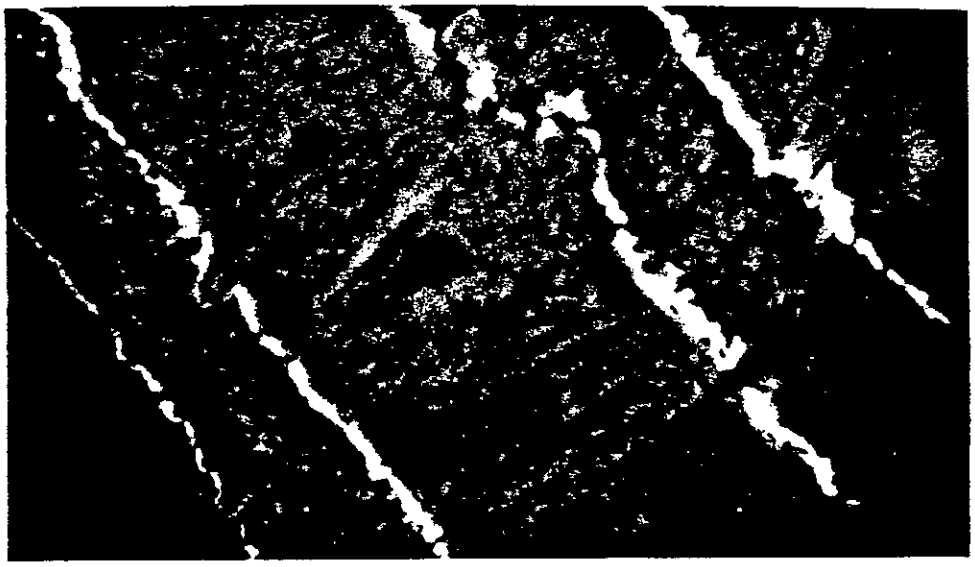


Plate 36 - Premaxillary suture; DCAF, 100x. Distinct fluorescence at bone surfaces similar to that seen for tetracycline.

Plate 37 - Premaxillary suture; tetracycline, 160x. Greater fluorescence of surrounding tissues.

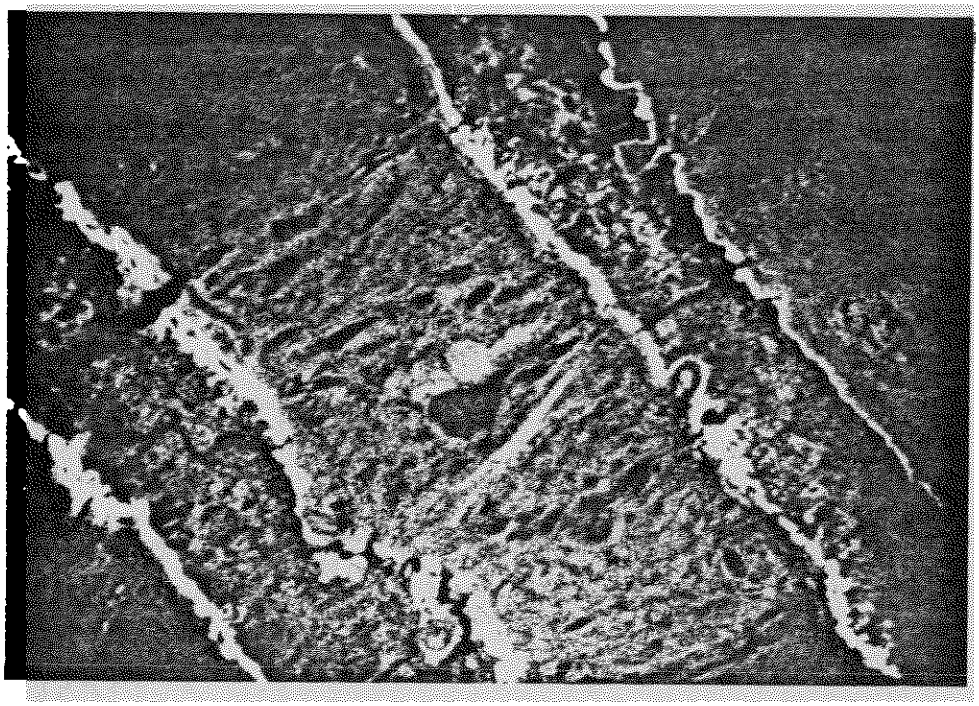
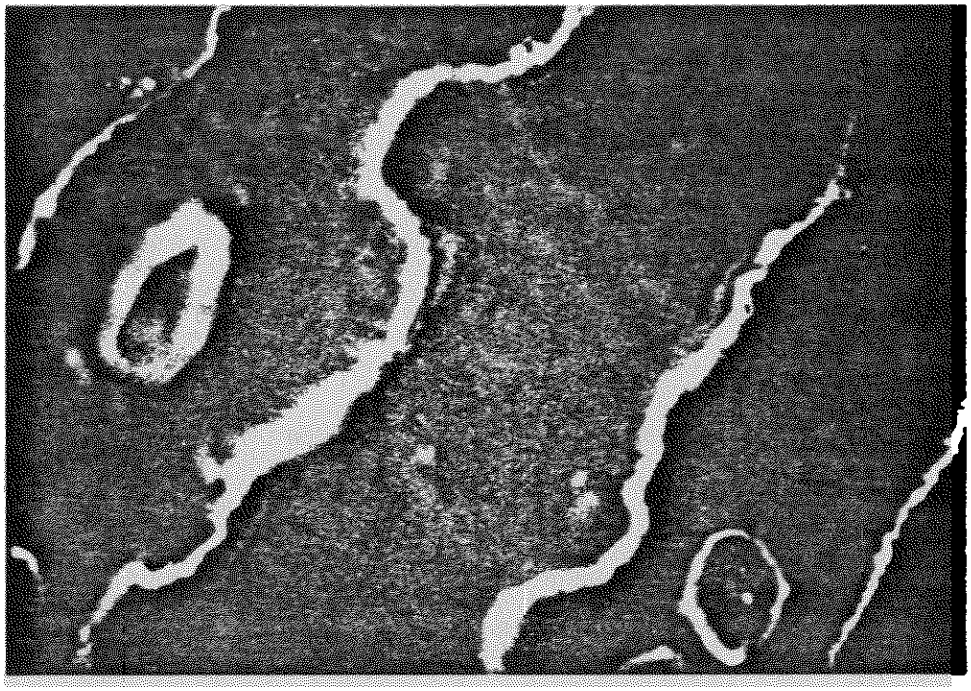




Plate 38 - Unstressed premaxillary suture; DCAF, 250x. Equal fluorescence is observed for all bone surfaces.

Plate 39 - Unstressed premaxillary suture, higher magnification; 400x. DCAF.



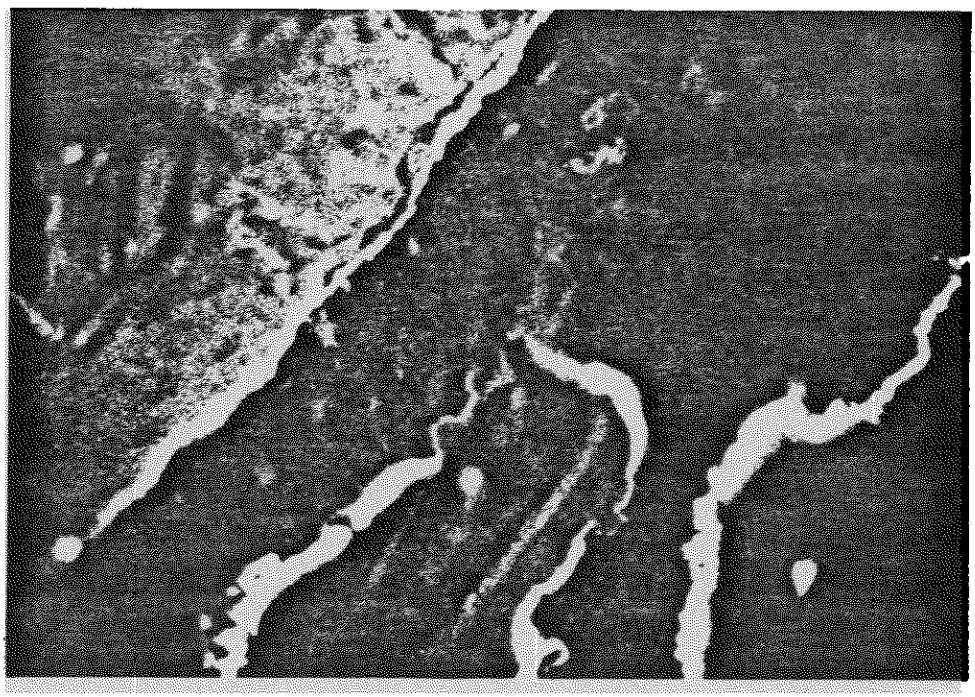


Plate 40 - Stressed premaxillary suture; DCAF, 250x. Thick band of fluorescence is observed adjacent to the suture. Minimal fluorescence is seen adjacent to the periodontal membrane.

Plate 41 - Stressed premaxillary suture, higher magnification; 400x. DCAF. Fluorescent band clearly distinguishes initially formed bone from subsequently formed bone.



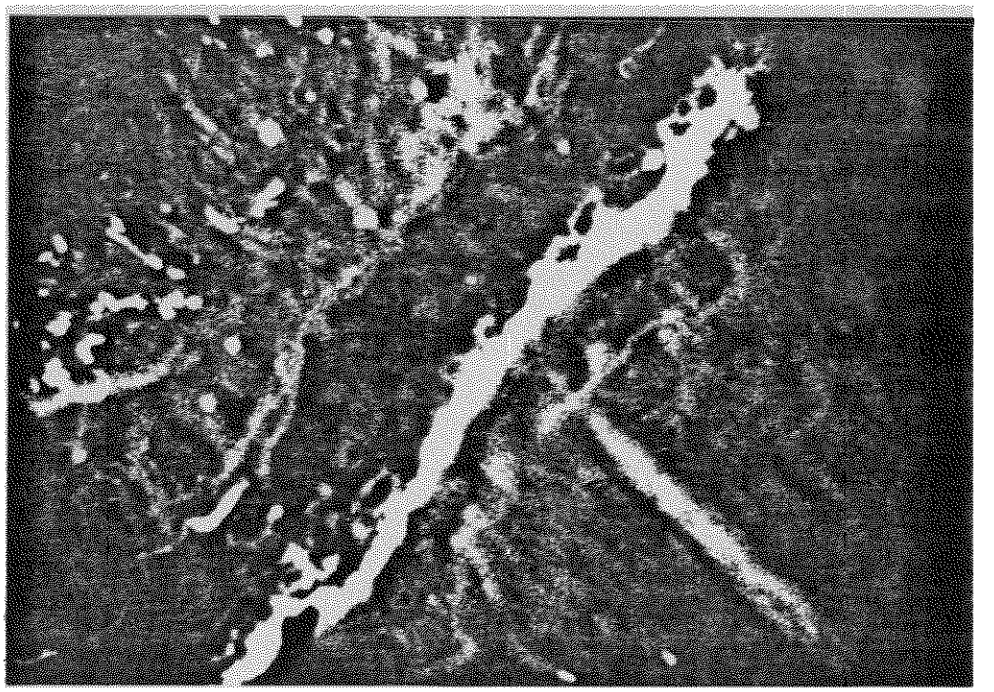
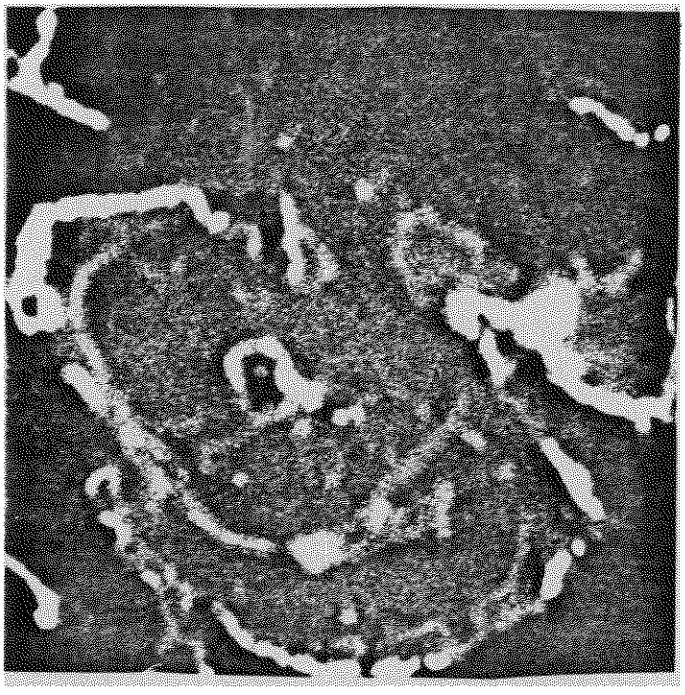


Plate 42 - Unstressed midpalatal suture; DCAF, 160x. Fluorescence visible on bone surfaces.

Plate 43 - Stressed midpalatal suture; DCAF, 160x. No differences in fluorescence.



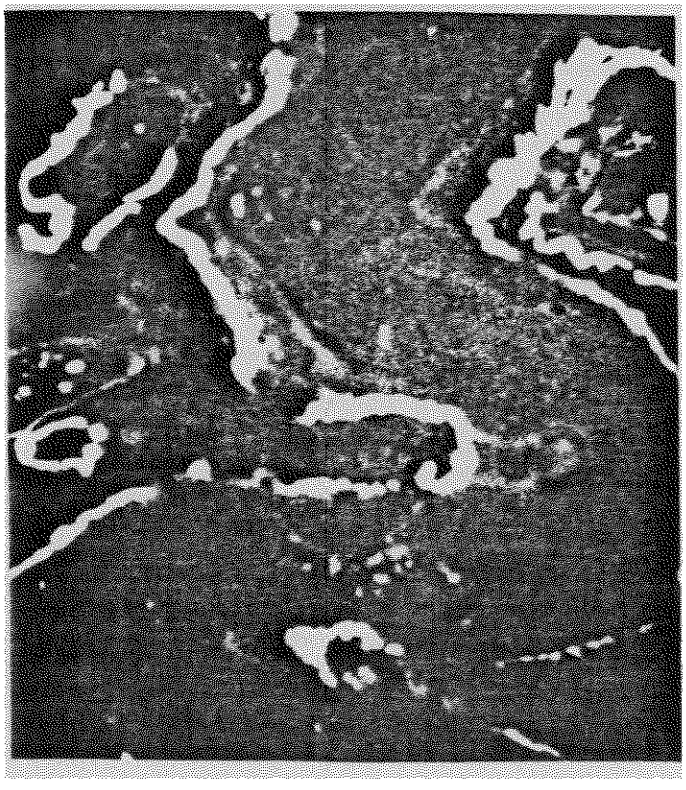
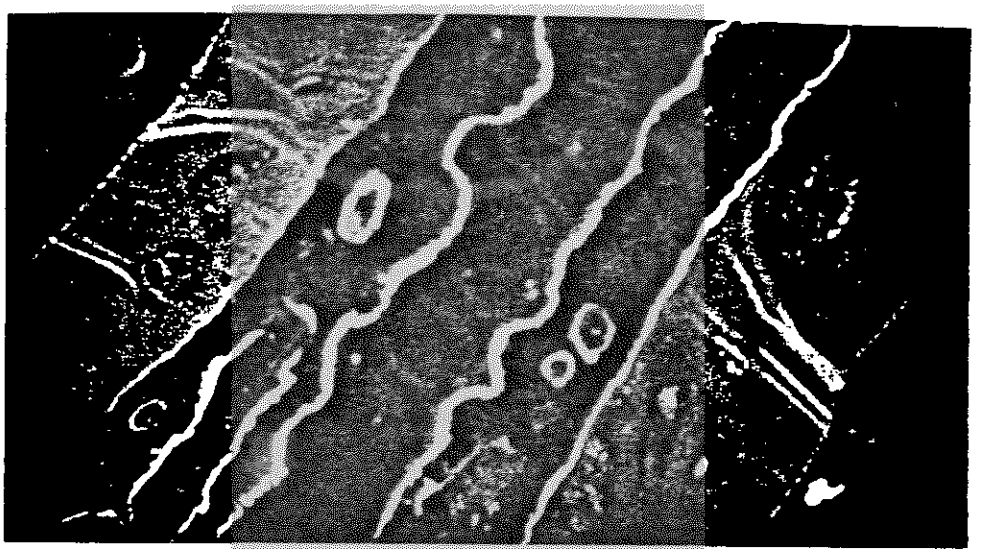
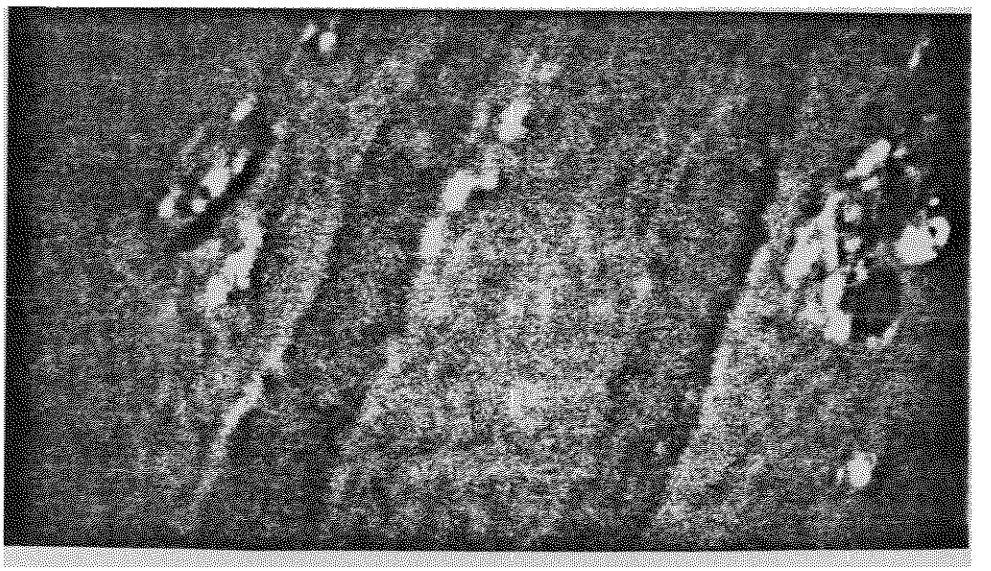


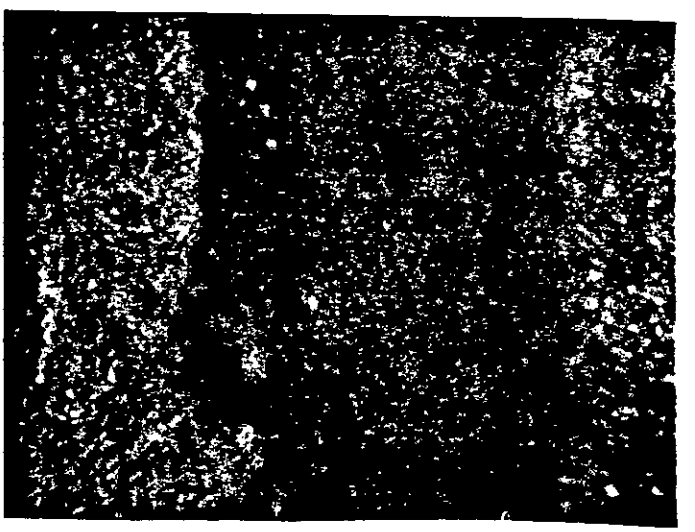
Plate 44 - Undecalcified premaxillary suture; DCAF, 100x. Typical fluorescence observed.

Plate 45 - Undecalcified premaxillary suture; UV control, 100x. Minimal auto fluorescence.

Plate 46 - Decalcified premaxillary suture; DCAF, 100x. Minimal fluorescence. DCAF is completely washed out.







DISCUSSION

The purpose of this study was to determine the distribution of BSA in bone during the remodeling process as a step toward understanding the significance of its presence. In addition, the localization of BSA was compared to two commonly used bone markers, tetracycline and DCAF, in order to elicit its role as a potential tracer of bone formation. An experimental period of three days was chosen because it was estimated that this was sufficient time to allow for the biochemical as well as histologic changes associated with bone formation. Related studies have used a similar time frame (Owen et al., 1973; Okamura, 1983). Deposition of iodinated albumin in bone has been demonstrated within one hour (Owen et al., 1973).

Other investigative groups have used an appliance similar to the intraoral coil spring utilized in this study (Storey, 1955; Stark and Sinclair, 1987). However, these previous studies used the appliance primarily to produce orthodontic tooth movement to investigate mechanical stress induced bone remodeling. This was accomplished by using animals of such an age that the premaxillary suture was closed. Because all surfaces of bone undergoing active remodeling in tooth movement consist of a mosaic of resorptive and depository fields (Enlow, 1982), the orthodontic tooth movement model does not allow good discrimination of pressure and tensile forces. The validity of the appliance system for clinical research, however, is not

questioned. In order to minimize this problem, rats used for the study were of such an age that separation of the premaxillary suture was still possible. What resulted was an effective model for stress induced bone remodeling. The premaxillary suture is relatively straight in comparison to the complex structures observed for many other cranial sutures. Use of this suture made orientation easier and enabled perpendicular sectioning. Therefore, the simplified model allowed for interpretation of experimental results with less difficulty.

Although the force delivered by the activated appliance was relatively small (15 g) an orthopedic response resulted causing suture separation with minimal tooth movement. This was confirmed by tetracycline and DCAF bone markers. While 15 g of force would certainly not elicit an orthopedic response in humans, optimum force guidelines for the rat model to control orthodontic versus orthopedic effects have not been established. Histologic results pertaining to this observation will be discussed later in this section.

It was felt that distant effects from the activated intraoral coil springs would be expressed across the midpalatal suture. In addition to the premaxillary suture, the midpalatal suture could act as a model for mechanical stress induced bone remodeling. Although active bone deposition was observed by fluorescence of tetracycline and DCAF under ultraviolet light, the midpalatal suture was extremely variable and often quite

complex. Thus, the midpalatal suture was not a good model for bone remodeling due to circumstances similar to that observed for the tooth model of stress induced remodeling.

Although the experimental design provided a simple anatomical model for mechanical stress induced bone remodeling, some deleterious effects were observed in regard to weight gain. While catch up weight gain over the course of a longer experimental period can only be speculated, it was apparent that an activated appliance interfered with the eating efficiency of the rats. Since placement of a passive appliance apparently had little effect, it can be inferred that the orthopedic response produced by the coil spring was responsible for slower weight gain. Nevertheless, normal behavior was observed for all animals regardless of placement of an activated appliance.

A modified technique utilizing a surgical procedure was necessary to insure proper intravenous injections. While no deleterious effects were observed due to the procedure in regard to weight gain, the overall effectiveness of the procedure must be questioned. Details of these questions will be presented later in this discussion.

Routine histologic results demonstrated separation of the premaxillary suture in animals receiving activated intraoral coil springs. Hematoxylin and eosin and toluidine blue stained sections provided information to correlate with findings from tetracycline and DCAF fluorescence. While half the specimens in

the study were demineralized prior to sectioning, lack of decalcification presented limitations on the information made available by routine histology. Small thin sections were used limiting the amount of tissue that could be seen at any one time. Due to the limitation in size, sections of the premaxillary suture and associated bone were necessarily taken near the crest of bone and could not be taken at greater depths. Undecalcified tissues were difficult to infiltrate with embedding material making bone difficult to section and enamel impossible. Thus, the periodontal membrane was often obscured in those sections especially near the tooth junction. In comparison, decalcified specimens were much more convenient to work with, lending themselves to visualization of suture, bone, periodontal membrane, and tooth structures including the pulp, dentin, and enamel. In addition, sections of the premaxillary suture could be taken at much greater depths in order to illustrate a more complete picture of the histology involved.

It should be noted that the technique employed in this study to prepare undecalcified specimens for observation had many advantages when compared to those commonly such as ground or mill sectioning. Cellular preservation was made possible by ultramicrotome sectioning using a diamond knife. In addition, consistent thickness of 1 μm were routinely achieved for these sections. Typical studies using ground or milled sections must deal with lack of cellular preservation and increased section

thickness in interpreting results.

Results from the immunohistochemical localization of BSA showed a light non-specific binding, identical to that of controls, indicating that BSA was not present. These results are inconclusive, however, as the technique is very sensitive. Antigen preservation is of utmost importance and conventional methods of tissue preparation for immunolocalization include frozen sectioning and paraffin embedding (Pearse, 1980). However, when mineralized tissues are involved these methods do not provide adequate preservation of tissue morphology. This includes specimens undergoing demineralization (Kapor, 1980). Thus, we utilized JB-4 for embedding. JB-4 is a highly purified glycol-methacrylate which gives better tissue preservation. It can be used for thin and thick sectioning and is easy to handle especially during immunostaining. In addition, it is water soluble making antibody incubation more effective. Regardless of theoretical advantages though embedding media remain a significant source of variation in immunohistochemical results.

Specificity of antibody for the target antigen is the most important factor for successful immunostaining. Despite using commercially available antigens and antibodies, the specificity of goat anti-BSA antibody for BSA was a potential source for the negative immunostaining that we observed. The possibility exists that the BSA antigen we used was not the same antigen used to generate appropriate antibodies. Our BSA was purchased

through Sigma, however was not prepared in Sigma laboratories. This particular grade was used because it was felt that it was of sufficient purity to attain specific immunostaining and would be cost effective. However, more highly purified versions of BSA were available and intended for highly sensitive radioimmunoassay procedures. This antigen may have been more suitable for use in our study.

ELISA was used to confirm antibody specificity for the BSA antigen supported by the microtiter plate. Although affinity curves were generated, the range of antigen concentrations that provided useful information was very narrow. A decrease in BSA concentration resulted in a decrease in absorbance. However, results from antibody dilution were variable and did not necessarily result in less absorbance. Moreover, the affinity curves did not resemble typical affinity curves (Engvall and Perlmann, 1971).

ELISA does not differentiate different antigen-antibody species in the system. Thus, it is possible that the antibody-antigen reaction observed in our results were due to contaminant antibodies reacting with impure antigen.

Impurities in our antibody solution were a potential factor in the negative immunostaining. Although no effort was made to solve this problem a potential solution would be to repurify the antibody solution utilizing affinity chromatography (Tsay and Veis, 1985). Fractions demonstrating good affinity could be

gathered, concentrated, and used for subsequent experiments.

Inhibition ELISA tested for cross reactivity between rat and bovine serum albumin. Results showed variable amounts of inhibition using rat albumin as the inhibitor. A typical inhibition curve could not be generated although significant inhibition was observed. Thus, cross reactivity could not be concluded although the results did nothing to ascertain that cross reactivity between rat and bovine albumin was improbable. Additional inhibition ELISA procedures utilizing structurally unrelated inhibitors may provide additional information.

The source of antibodies is a potential source of negative immunostaining. Antibodies developed in goat have a poor track record in comparison to the same antibodies generated in rabbit. This is especially true for the albumins.[1] Unfortunately, the only commerically available antibodies to albumin at the time of this experiment were developed in goat. Anti-albumin antibodies are being developed in rabbit and experimentation with these antibodies may provide more useful information.

Because indirect immunochemical techniques were utilized in the experiments, a second antibody was employed to produce a visible color reaction and also amplify the result by multiple binding. This second antibody may be an additional source of negative immunostaining. Non-binding of the enzyme conjugated

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[1]Personal communication with Leigh Gaskill, Sigma technical services.

second antibody to the first antibody would pose similar problems discussed for the first antibody. No attempt was made to characterize the second antibody or evaluate it as a potential source of negative immunostaining.

Proper administration of BSA into the rats was critical for successful localization of BSA in target tissues. A modified procedure utilizing a surgical technique was employed for intravenous administration of BSA. Although the technique was successful using tetracycline in a pilot study, it was apparent that consistency of results would pose a potential problem. The small size of the vein and its inherent friability made successful injection very difficult even though visibility was greatly enhanced by the surgical procedure. Inconsistent injections of BSA would ensure negative immunolocalization of BSA at the target tissues.

The immunohistochemical localization of endogenous rat albumin was studied to confirm its presence in calcified tissues. No attempt was made to correlate these findings with the possible localization pattern during active remodeling. This role was reserved for BSA. The immunostaining procedure confirmed the presence of endogenous albumin ubiquitously. The results must be interpreted with caution, however, as similar limitations are posed in the study of endogenous albumin as those observed for BSA. ELISA was employed to confirm the specificity of anti-rat albumin antibody for the rat albumin

antigen. Although decreasing concentrations of antigen produced less absorbance, antibody dilution resulted in extremely variable results and did not result in less absorbance in many cases. In addition, albumin from animals used in the study was not isolated and purified to serve the role of antigen in subsequent tests. Rather, commerical sources of rat albumin were used. This may have provided an additional source of inaccuracy.

As expected, administration of tetracycline and DCAF resulted in fluorescence under ultraviolet light when undecalcified sections were observed. However, decalcified sections were totally devoid of any fluorescent illumination and were identical to controls not receiving injections of either bone marker. Thus, the tetracycline and DCAF were completely washed out during demineralization. The usefulness of these vital bone markers is limited to undecalcified specimens. The difficulty in handling these tissues has been discussed previously.

The results obtained for tetracycline and DCAF fluorescence correlated well with routine histologic results in regard to the location and pattern of newly formed bone. Application of an activated intraoral coil spring resulted in greater bone deposition on the suture side of bone as opposed to the periodontal membrane side. This was demonstrated by routine histology and confirmed by fluorescence. Although bone

deposition on the suture side of bone was not unexpected, it was surprising that bone deposition was less conspicuous on the periodontal membrane side of the same bone. Since the appliance produced laterally-directed force to the maxillary central incisors and across the premaxillary suture, tension would be produced in the periodontal membrane as well as the suture. Thus, according to the pressure-tension hypothesis of Reitan bone deposition would be expected on either side of the bone. Perhaps threshold levels of force necessary to produce bone deposition were achieved on the suture side, but not on the periodontal membrane side of bone. In addition, the distribution of osteogenic cells may be greater in the suture than in the periodontal membrane. Furthermore, the possibility of plastic bone deformation as described by Baumrind cannot be ruled out.

SUMMARY AND CONCLUSIONS

The noncollagenous proteins of bone extracellular matrices have attracted much attention in regard to their potential importance in the phenomenon of bone remodeling. Albumin, a plasma-derived noncollagenous protein of bone, is present in extravascular sites in all connective tissues which have been investigated. Preliminary investigations have indicated that serum albumin is present in the calcified bone matrix and may be associated with the mineralization front during enamel and dentin formation.

The present study was undertaken to investigate the localization of albumin in bone tissue to better understand the significance of its presence. In addition, the immunolocalization of albumin was compared to two commonly used bone markers, tetracycline and DCAF, to study its potential role as a bone tracer.

The study utilized twenty Sprague Dawley rats which were approximately 40 days old. One half of the animals received a standardized intraoral coil spring designed to exert a lateral force of 15 g to the maxillary central incisors and across the premaxillary suture. Controls received a passive coil spring or nothing at all. All animals received one of the following injections: intravenous phosphate-buffered saline (for controls), intravenous bovine serum albumin (1.5 g/kg), intraperitoneal or intramuscular tetracycline (30 mg/kg), or

intraperitoneal DCAF (15 mg/kg). A modified procedure utilizing a surgical technique was employed for intravenous injections.

At the end of the three day experimental period, maxillary central incisors and surrounding bone and mid-palatal suture were surgically removed and processed for histology. Half of the specimens were decalcified in EDTA prior to embedding. BSA sections were stained by an indirect immunoperoxidase technique. In addition, endogenous rat albumin was localized by similar methods. Tetracycline and DCAF sections were observed under ultraviolet light.

The experimental design utilizing an intraoral coil spring provided a simple anatomical model for mechanical stress induced bone remodeling. Although the premaxillary suture served this purpose well, the mid-palatal suture was too variable and complex to be considered an effective model. The appliances were well-tolerated, but did result in diminished weight gain when activated. No deleterious effects were observed as a result of the surgical intravenous injection procedure.

Routine histologic results were obtained for both undecalcified and decalcified sections. Undecalcified specimens were much more difficult to handle and limited the amount and quality of tissue seen under one view.

Results from indirect immunoperoxidase localization of bovine serum albumin showed light non-specific binding indicating the absence of bovine serum albumin in target

tissues. Endogenous rat albumin was present ubiquitously as shown by similar methods.

Results from immunostaining must be interpreted with caution. ELISA and inhibition ELISA were inconsistent in evaluating antibody affinity. In addition, the sources of commerical antigens and antibodies are questionable. Antibody purity could not be assured and cross reactivity between rat and bovine albumin could not be ruled out.

The results of this study in regard to immunostaining are inconclusive and suggest that the technique for localization of exogenous serum albumin is in need of refinement.

Tetracycline and DCAF were effective vital bone markers and results from undecalcified sections correlated well with routine histologic findings. However, demineralization of specimens caused these bone markers to be completely washed out, thus, limiting the usefulness of these compounds to undecalcified specimens. The technique employed in studying undecalcified specimens was very effective when compared to results from ground or mill sectioning. Cellular preservation was good and fluorescent banding sharp due to consistent thin sectioning with a diamond knife. Results from tetracycline and DCAF fluorescence confirmed routine histologic findings that greater bone deposition was produced adjacent to the suture by application of an active appliance, and the intensity was markedly reduced on the periodontal membrane side of the same bone.

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