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EFFECTS OF BETA-AMINOPROPIONITRILE ON RAT MOLAR
PERIODONTAL LIGAMENT, BONE MATRIX FORMATION
AND ITS MINERALIZATION

BY

HARINDER S. SANDHU, B.D.S.

A THESIS

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CHAPTER 1.

INTRODUCTION

1.1 LATHYRISM

1.1.1 Historical Background

Lathyrism is a nutritional disease produced as a result of ingestion of certain plants of leguminaceae family or their toxic constituents by many animals including man. The lesions thus produced are mainly skeletal and neurological. The history of deleterious lesions produced by sweet peas in humans, goes back to ancient times. Hippocrates described these effects as "All the men who ate sweet peas became impotent in legs". The term Lathyrism was introduced by Cantani (1874) in Italy who attributed these effects to some nutritional factors. Schuchardt (1885) was the first to review the literature and describe the neurological lesions produced by Lathyrus sativus (Kesri Dal) in India. There are many reports of epidemics of lathyrism from France, India, Russia, Spain and Syria. All of these coincided with famine situations due to wheat crop failures and consumption of sweet peas as a staple ingredient of human diet. Apart from L. sativus (chick peas), both L. cicera (flat podded velch) and L. clytenuum (Spanish velching) have been implicated in this nutritional condition. These three species are relatively non-toxic to other animals. Another species (L. latifolus) however produces neurological symptoms in rats which resembled human lathyrism (Liener, 1966).

The rats fed with L. odoratus (Sweet peas), L. pusillus (singletary peas), or L. hirsutus (Caley peas) develop marked

skeletal deformities that were quite different in symptomatology from human lathyrism.

1.1.2 Isolation of toxic factor from sweet pea seeds

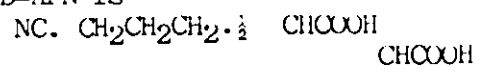
The Isolation of the active principle in purified form, capable of producing the symptoms of osteolathyrism was first reported by Dupuy and Lee in 1954 from L. pusillus. Soon after the lathyrigen was also isolated from sweet peas (Dasler 1954; McKay et al., 1954).

The chemical structure of the toxic agent as B (N-γ-L-glutanyl) amino propionitrile was determined by Schilling and Strong (1954, 1955). Further investigation discovered that the γ-glutamyl portion of the molecule did not have any lathyritic activity and that it was the B-aminopropionitrile segment, commonly abbreviated as B-APN, which was responsible for the this deliterious effects on bones (Ponseti and Shephard, 1954, Dasler, 1954, 1954. Bachuber et al., 1955, Wowzonek et al., 1955).

1.1.3 Physico chemical Properties of the Lathyritic factor

B-APN is a white, odourless, crystalline powder with a melting point 172° - 173°C and produced for the first time by Abbotts of North Chicago, Ill., U.S.A. as B-APN fumarate. It is water solubule and crystallizes with 80% ethanol and is stable at room temperature.

Chemical Formula of B-APN is



with a molecular weight of 128.2.

1.1.4 Metabolism of B-APN

When ^{14}C labelled B-APN is given to rats about 80-90% of the total administered activity appears in urine within 24 hours. Of this about 40% is unchanged and is excreted as B-APN, while another 25-30% is eliminated as cyanoacetic acid (Lalich, 1958). A high level of radioactivity is also seen in various organs such as liver and epiphyseal plate cartilage (Ponseti et al., 1956).

Lalich in 1958 reported that the detoxification of B-APN involves an amine oxidase. Kulonen in 1961 reported that the amino group of B-APN is essential for its activity and confirmed earlier findings of Lalich, (1958) that the detoxification involved deamination. Pargyline HCl, an inhibitor of the enzymes monoamine oxidase, blocked the detoxification of B-APN and also reduced the disappearance of the lathyrogen from the body (Keiser et al., 1967).

1.1.5 Lathyrogens as Teratogens

The first Teratogenic effects of the lathyrogens were reported by Tourney et al., (1943). They described the resorption of rat fetuses in mothers feeding on L. odoratus. Since then many investigators have studied the teratological effects of sweet peas in mammals (Stanler, 1955; Writschafter and Williams, 1957; Telford et al., 1962; Keeler et al., 1967; 1969, Steffek, 1969, and Steffek and Hendrickx, 1971; Barrow, 1971; Steffek et al., 1971; Barrow and Steffek, 1974).

A majority of these teratological studies have been conducted on rats (Herd and Orbison, 1966, Barrow, 1971, Steffek et al., 1971, Pratt and King, 1972, Barrow and Steffek, 1974, Mato et al., 1975) and the most commonly produced abnormalities in fetuses from mothers treated with lathyrogens during pregnancy are cleft palate (Writschafter and Williams, 1957; Herd and Orbison, 1966, Abramovich and Devoto, 1967, Steffek et al., 1971, Pratt and King, 1972; Matto et al., 1975; Walker, 1975, Diewart, 1980), ectocardia and gastroschisis (Barrow, 1971; Barrow and Steffek, 1974), and osteopathologies (Fern, 1960).

The period of active organogenesis is the critical period and most of the teratogens act during this period to produce the various abnormalities. As the period of active organogenesis passes the susceptibility of the embryos to teratogens decreases (Wilson, 1973). Barrow and Steffek, (1974) have described B-APN as not a typical teratogen capable of causing malformation in early organogenesis. Their findings are in agreement with earlier reports that lathyrogen-induced teratogenic effects, in rat and mouse, occur relatively late in gestation (Herd and Orbison, 1966, Steffek et al., 1971).

Wiley and Joneja, (1976) conducted a comprehensive study on Teratogenic effects of B-APN (active principle of L. odoratus) in Golden Syrian Hamster and concluded that B-APN is a true teratogen in this species. B-APN, as an active teratogen, has also been shown effective in some avian species (Rosenberg, 1957;

Zachin and Goldhaber, 1959, Cameron, 62 a, b, Hall, 1972). It has also been shown that different animal species have variable sensitivity to L. odoratus (Barrow et al., 1974).

1.1.6 Osteolathyrism and Neurolathyrism

These terms were coined by Hans Selye, (1957) to describe the effects of a diet containing peas of the genus Lathyrus on osseous and nervous tissue respectively.

About two and a half decades before Selye introduced these terms, Geiger et al.. (1933) had described the effects of sweet peas (L. odoratus) on bones. They observed lower bone ash contents, abnormal spinal curvatures, sternal deformities, enlargement of costochondral junctions and curved bones. These findings were confirmed subsequently by numerous other authors (Lewis et al., 1948; Ponseti and Shepard, 1954, Selye, 1957; Menzies and Mills, 1957, Tanzer, 1965, Alco, 1969, Selye, 1970).

Other changes produced by B-APN on the skeletal system are loosening and detachment of tendinous and ligamentous insertions, widening and extensive disruption of the epiphyseal plates, detachment of tibial tuberosity, subluxation and dislocation of shoulder joint, diastasis of sacroiliac joints, degeneration of intervertebral discs and disc herniation (Ponseti and Shepard, 1954). To this list of abnormalities produced by sweet peas were added dissecting aneurism by Ponseti and Baird, (1952).

As mentioned earlier the active principle for osteolathy-
rism was shown to be B-APN. However there are other lathyrogens
that produce similar osteopathologies. These compounds are
amino-acetonitrile (Aan), D-Pencillamine (Pen), semicarbazide
(Sc), and cystamine dihydrochloride. The mechanism of their
action on collagen, however, differs from that of B-APN as well
as from each other.

Neurolathyrism is characterized by relatively acute onset
of weakness in the legs preceded by pain and parasthesias
(Paissos and Domopoulos, 1962; Gardner and Sakiewich, 1963).
The end result is a permanent spastic paraplegia of the lower
extremities characterised by increased muscular tonus, increased
deep tendon reflexes, a positive Babinski sign, and absence of
nerve regeneration. The lathyrogens producing these neurologi-
cal symptoms do not contain B-APN and are L. latifolius, L.
sativus and L. cicera. Reesler et al., (1961) and Bell, (1962)
have isolated L-, γ -diaminobutyric acid as the active agent of
L. sativus. (Adiga et al., 1963, Mutri et al., 1964; Rao et
al., 1964). However the mechanism of their action in producing
neurolathyrism is still obscure (Barrow et al., 1974).

1.2 Mechanism of Action of Osteolathyrogen

Soon after the demonstration by Dasler (1954) that B-APN affected the tissues with high collagen and elastin contents, an extensive search began to find the mechanism of action of the toxic agent.

1.2.1 Effects on Collagen and Elastin

Martin et al., in 1961 reported that B-APN inhibits the inter and intra molecular cross linkages of collagen during the extra cellular maturation of collagen fibrils. Page and Benditt in 1967, attributed this effect of B-APN to its inhibition of the enzyme lysyl oxidase, which is responsible for the initial step in both collagen and elastin fibre formation. The oxidative deamination of peptide bound lysine and hydroxylysine residues in the tropo collagen molecule is mediated by lysyl oxidase. This oxidative deamination results in the formation of Σ -semialdehyde of L-amino adipic acid (allysine), and the Σ -hydroxy, Σ -semialdehyde of L-amino adipic acid (hydroxylysine) (Barrow et al., 1974). Aldol type cross-links formation is the next step and the product is known as Aldol Condensation Product (ACP) (Rojkind et al., 1968). These links formed by condensation of aldehydes or between aldehydes and Σ -NH₂ groups of lysyl or hydroxylysyl residues result in the formation of aldimine type of cross links.

In the case of elastin also B-APN inhibits the oxidative deamination in a similar fashion (Page and Benditt, 1967).

The formation of these cross links is dependent on initial deamination mediated by lysyl oxidase. This is a critical step in the formation of aldehydes and subsequent production of cross links. As the ability of collagen and elastin to act as a major structural component of many tissues is dependent upon these covalent cross links (Peacock, 1966) the B-APN inhibition of enzyme lysyl oxidase undermines the structural integrity of collagen. The collagen thus produced is very deficient in tensile strength (Barrow et al., 1974).

The mechanism of action of all lathyrogens on collagen is not through inhibition of enzyme lysyl oxidase. D-pencillamine acts by forming complexes with preformed aldehyde groups and thus preventing the formation of Aldol Condensation Product (Deshmukh and Nimni, 1969; Pinnel et al., 1968; Gallop and Paz, 1975).

1.2.2 Effects on Cartilage

As the collagen is a major constituent of cartilage the effects of lathyrogens were also studied in this tissue. Most of the investigators agreed with the cross link defect hypothesis. The major changes under B-APN influence were reduction in total number of collagen fibrils (Follis and Tousimis, 1958) and their mean diameter (Matukas et al., 1968; Wiley and Joneja, 1976). The vacuolization of chondroblast mitochondria was the most prominent cellular effect of B-APN described by Wiley and Joneja, (1978).

1.2.3 Effects on Bone

Though it was known since 1933 (Geiger et al., 1933) that B-APN produces changes in bony structures, the internal changes in bone produced by B-APN were obscure. The similarity of experimentally induced osteolathyrisms to Legg-Calvé-Perthe, disease, Paget's disease, adolescent kyphosis, and certain idiopathic skeletal abnormalities led many investigators to begin a search for the mechanism underlying the osteolathyratic defect (Ponseti and Shepard, 1954). High content of collagen in bone was a major reason that most of the studies concentrated on the fibrillar component of the bone and the cartilage matrix, (Matukas et al., 1967, Wilcy and Joneja, 1976).

The covalent cross links of collagen were considered a prerequisite for collagen fibrils to attract mineral in bone (Mills and Bavetta, 1968; Avioli, 1973). Barrow et al., (1974) as a result of extensive review of earlier work as well as on the basis of their own findings concluded that most of the osteopathologies produced by B-APN were due to its inhibition of inter- and intra molecular cross linkages in collagen.

Many workers investigated the effect of B-APN on the non-collagenous component of connective tissue matrix. There appears to be a major conflict between these reports, some contending that the component increases with B-APN treatment (Churchill et al., 1955, Menzies and Mills, 1957; Writschatter, 1957, Gillman and Hathorn, 1958; Belanger, 1958, Blokely and

Orbison, 1964) while others insist on their decrease (Pyörälä et al., 1957, Rammurti and Taylor, 1958, Karnovsky and Karnovsky, 1961, Kennedy and Kennedy, 1962; Shintani and Taylor, 1962 a, b; Vanden Hoof, 1963; Picard et al., 1966, Reddi and Sullivan, 1979).

There have also been some reports on cellular toxicity of B-APN on adult tissues. These changes include degeneration of osteoblasts (Clemmons, 1957), retrogressive changes in fibrocytes (Dasler and Milliser, 1957), cytoplasmic vacuolization of fibroblasts in vitro (Levene, 1966) Chromatolysis (Wirtschafter, 1957) and vacuolization of chondrocyte mitochondria (Wiley and Joneja, 1976). In a physiological study Juva et al., 1959, demonstrated that B-APN depresses the consumption of O_2 by liver and cartilage slices in vitro.

1.3 BONE AS A TISSUE

1.3.1 Bone Cells and Bone Matrix

Bone is a highly specialized form of connective tissue where the organic matrix is highly mineralized. The latter is largely composed of calcium and phosphate as apatite crystals together with small amounts of other inorganic elements.

Bone has two main types of cells, formative cells and resorptive cells. The precursor of formative cells are undifferentiated mesenchymal cells, the osteogenic cells, which line the deepest layers of periosteum. The osteogenic cells differentiate to form bone forming cells, the osteoblasts (Kember, 1960, Young, 1962, 1963, 1964; Owen, 1963, '65, '67, '70, '71; Owen and Macpherson, 1963, Bingham et al., 1969). The osteoblasts form a complete covering over all the bone surfaces. They control the movement of calcium and phosphate between the bone and the blood (see later page no. 18). They synthesize and secrete all the organic components of the matrix that includes collagen (Carneiro and Leblond, 1959) and protein-polysaccharide complexes (Lea and Vaughan, 1957, Leblond and Weinstock, 1957; Owen and Shelter, 1968). The ultrastructure of osteoblasts present the picture of a cell which is very actively synthesizing proteins and glycoproteins, with a very well defined endoplasmic reticulum and golgi apparatus (Jande and Belanger, 1971, 1973, Jande, 1972).

There is an increasing body of evidence that osteoblasts

are also involved in the calcification of matrix they themselves synthesize (Bernard and Pease, 1969; Bonucci, 1971, Matthews et al., 1971; Anderson and Reynolds, 1973). As the osteoblasts form matrix they become enclosed in lacunae, and become osteocytes. Bélanger, (1971) has described them as imprisoned osteoblasts. Various stages in the life cycle of an osteocyte are well documented (Jande and Belanger, 1971, 1973). There are indications that osteocytes are metabolically active at least in their initial life (Young, 1962, Owen, 1963; Jande, 1972, Jande and Belanger, 1973).

The second group of cells which are resorptive in function are known as osteoclasts. These are very large multinucleated cells, very rich in hydrolytic enzymes like acid phosphatase (Ham and Cormack, 1979). By very ingenious experiments (Walker, 1975) it has been established that they are derived from the monocyte line of cells in the bone marrow. The fine structure of osteoclasts has been extensively studied (Cameron, 1972; Doty et al., 1972; Luk et al., 1974, King et al., 1978).

The organic component makes about 30% of total bone matrix (Eastoe, 1956). Its major constituent is the fibrous protein, collagen (Glimcher and Krane, 1968) which is very rich in glycine and proline (Udenfriend, 1966). The non-collageous component of organic bone matrix include glycoproteins (Herring, 1972, Ashton et al., 1974), bone sialoprotein (BSP) (Andrew and Herring, 1965), albumin (Owen and Triffitt, 1972; Owen and

Melick, 1973) and lipids (Wuthier and Irving, 1964; Wuthier, 1966, 1968; Wuthier and Shapiro, 1966).

1.3.2 Inorganic Components of Bone

Seventy percent of the bone matrix is formed by inorganic salts. Hydroxyapatite (Woodard, 1962; Eanes and Posner, 1965), and amorphous calcium phosphate (Robinson and Watson, 1955, Hancox and Boothroyd, 1966; Harper and Posner, 1966; Termine and Posner, 1966) are the major constituents of the inorganic part of bone matrix. Other constituents in smaller amounts are fluoride (Zipkin et al., 1963), sodium (Davies et al., 1952), potassium (Triffitt et al., 1968), magnesium (Foster, 1968), strontium (Bennit, 1972), citrate (Dixon and Perkins, 1952) and some other trace elements.

1.3.3 Mineralization of Bone

The organic matrix formed by the osteoblasts undergoes mineralization. The details of mechanism of the mineralization are not agreed upon by all researchers. The following theories present various diverse view points.

(i) Direct Deposition Theory

The collagen as a template for nidation of mineralization was the prime target of early researchers of mineralization process. The indirect evidence concerning the relationship of bone the mineral to collagen fibrils of the matrix came from a polarizing light microscopic study which showed that mineral phase in calcified tissue is very highly organized

Rouiller, (1956) suggested a similarity of organization between the collagen fibrils and the inorganic mineral phase. Similar results were obtained by x-ray diffraction studies (Glimcher, 1959; Glimcher et al., 1965). With the advent of transmission electronmicroscopy (TEM) many researchers attempted to visualize the relationship between organic matrix and inorganic mineral phase of the bone. The observation of TEM showed direct relationship of minerals with the collagen fibrils of the bone matrix in different species (Fitton-Jackson, 1957, Robinson and Watson, 1952, 1955; Cooper et al., 1966). A close relationship of minerals and periodicity of collagen fibrils (700 Å) has also been cited as evidence for intimate association of the inorganic material and collagen fibrils (Robinson, 1952, 1955). Boyd, (1972) with the help of scanning electronmicroscopy showed that a major portion of the inorganic phase of the bone lies within the collagen fibres.

The studies by Glimcher and his associates were based upon the assumptions that:

- (i) Collagen in tissues which do not normally mineralize is prevented from reacting with calcium and phosphate ions by polysaccharides.
- (ii) in vitro calcification of tendon collagen is comparable to calcification of bone collagen which in vivo is complexed with lipo-proteins, protein-polysaccharides, protein-protein complexes, salt and water coupled with basic assumption that

collagen acts as a nucleation substrate for the formation of apatite crystals (Glimcher and Krane, 1968). A set of cross linkages associated with hexose and ester phosphate groups, were considered the localizing factor (Veis and Schlueter, 1964; Krane et al., 1965). The cross linkages were described as pre-requisites, for collagen fibrils to become mineralized (Mills and Bavetta, 1968; Avioli, 1973). The theory of collagen as a nidation site for minerals lost support when the direct evidence received from transmission electronmicroscopy in its favour was challenged. Many investigators on the basis of improved technique showed that deposits of the so-called mineral crystals on collagen fibrils shown by Fitton-Jackson, (1957) were in reality artefacts (Dudley and Spiro, 1961; Decker, 1966; Bernard and Pease, 1969). Boyd (1972) himself admits that a certain percentage of the mineral phase is in between the collagen fibrils and not entirely inside the collagen fibrils. Urist, (1976) has described the relationship of collagen fibrils with minerals as one in which collagen fibrils are not the cause of mineral deposit in bone but the arrangement is the result of a process of mineral deposits on and around the collagen fibrils, because of some other factors. The contention that the collagen fibrils are the initiating site for the mineralization was further challenged when the apatite crystals in the osteoid and cartilage were demonstrated to be not inside or on collagen fibrils but rather

inside membrane bound vesicular structures, the matrix vesicles. (Anderson, 1967, Bonucci, 1969; Bernard and Pease, 1969, Bonucci, 1971).

(ii) Matrix Vesicle Theory

Much recent evidence points towards the osteoblasts playing a major role in mineralization of bone matrix. Since Anderson, (1967) and Bonucci, (1967), first described matrix vesicles in calcifying cartilage, it has been found that these vesicles are also present in the bone at the site of first mineral deposits (Bernard and Pease, 1969; Bonucci, 1971, Anderson, 1973).

The matrix vesicles are trilaminar membranous vesicular structures of 300 Å to 1 μm in diameter (Anderson, 1976). These vesicles originate by pinching off from the distal portions of microvilli like processes of cells in calcifying tissues. In the cartilage they originate from hypertrophic chondrocytes and are present in groups inside the matrix. In the bone, they originate from osteoblasts and are present in the osteoid matrix. They are also present in smaller numbers around young osteocytes (Anderson, 1967, 1969, Bonucci, 1967, 1971, Anderson et al., 1970; Glauert and Mayo, 1973, Rabinovitch, 1974; Ornoy et al., 1980). The presence of certain enzymes like alkaline phosphatase and ATPase, a characteristic of osteoblast and chondroblast membranes, in the membranes of matrix vesicles further substantiate the above claim (Matsuzawa and Anderson, 1971). Matrix vesicles have been shown to contain lipids (Peres

et al., 1971, 1974, Wuthier, 1973), neutral polysaccharides (Bonucci, 1970), and amorphous calcium (Anderson et al., 1970, Ozawa et al., 1972, Brighton and Hunt, 1974; Gay and Schraer, 1975; Gay, 1977).

Dodds (1932) and Schenk et al., (1967), with the help of light and electronmicroscopy, have shown that longitudinal septa in epiphyseal plates undergo calcification while the transverse septa do not show mineralization. The conclusive evidence for the involvement of matrix vesicles in the calcification process was provided by Anderson, (1969) by demonstrating the presence of matrix vesicles exclusively in the longitudinal septae of epiphyseal plates. He further showed for the first time with the help of electronmicroscope, the presence of apatite crystals inside the matrix vesicles.

Ali and his associates in the United Kingdom have further shown that isolated matrix vesicles are capable of initiating calcification in vitro (Ali, 1976).


In the last few years there has been a flood of information regarding the role of matrix vesicles in initial mineralization. With the help of TEM, SEM and biochemical studies on healing alveolar bone sockets of dogs, it has been shown that matrix vesicles are involved in the initial mineralization (Sela et al., 1978; Sela and Bab, 1979). Similar results have been reported in calcifying human tendon by Sarkar and Untoff, (1978); in rat bone (Ornoy et al., 1980); in epiphyseal plates

(Levy et al., 1980), and woven bone (Martino et al., 1979).

According to Anderson (1973) the mineralization, in matrix vesicles, takes place in two phases. In the first phase calcium is concentrated into matrix vesicles by virtue of a lipid-calcium interaction and possibly by active transport of calcium ions across the vesicular membrane. The phosphate accumulates within and at the surfaces of matrix vesicles through the enzymatic hydrolysis of phosphate esters. The accumulated phosphate reacts with locally increased calcium to form apatite. This sequence of events is also supported by Ali (1977). During the second phase the initially formed apatite crystals become exposed to extra vesicular cartilage or bone matrix fluid by rupturing of vesicular membrane (Anderson, 1976). These crystals thus formed proliferate because the cartilage and bone matrix fluids under physiological conditions are saturated with respect to apatite crystals (Howell et al., 1978): It is in the second phase where the matrix factors such as proteoglycans, collagen, concentration of Ca^{+2} and PO_4^{-3} , and the hydrolysis of PPI would be expected to influence the rate of mineral proliferation (Anderson, 1976).

1.3.4 Bone Membrane

All the bone surfaces are known to be lined by a layer of cells of the osteoblasts family (Howard, 1959, Baud, 1968; Vitalli, 1968). Newman and his associates were the first to give a definitive evidence that the bone surfaces are lined by a



continuous layer of cells which separates the bone fluid compartment from primary extracellular fluid compartment (Terepka et al., 1968, Triffitt et al., 1968, Canas et al., 1969, Geisler and Newman, 1969; Newman and Mulryan, 1969). On the basis of his studies with parathormone and thyrocalcitonin on the movement of calcium ions necessary to maintain plasma calcium level, Talmage and his associates have postulated that all the bone surfaces are lined by osteoblasts, anatomically arranged so as to form a membrane whose function is to facilitate the movement of calcium to and from bone surface (Talmage, 1969, 1970; Talmage et al., 1970). Doty and Robinson (1970) have ascribed a specific role to osteoblasts of this bone membrane in mineralization of the bone matrix. In a detailed gerontological study it has been shown that the integrity of this bone membrane is very critical to the normal accretion and resorption process which is under cellular control (Tonna, 1978).

1.3.5 Enzymes Related to Bone Mineralization

(i) Alkaline Phosphatase

This is an enzyme which has a wide spread distribution and hydrolyzes the glycerophosphate and other monoesters of phosphoric acid at an alkaline pH. It is found in large quantities in bone, kidney and intestine.

Since Robinson (1923) suggested a role for alkaline phosphatase in the process of mineralization of bone, it has been a topic of discussion in numerous research communications (Robinson,

1923, Bourne, 1944, Lorch, 1949; Johnson, 1950; Follis, 1950, McLean, 1950, Siffert, 1951; Pritchard, 1952; Tonna, 1958, Ali et al., 1971; Anderson, 1973; Meikle, 1974; Anderson, 1976, Skillen and Rehmani-Nobar, 1980). There are two main views about its role in the bone metabolism. Some workers believe that its presence at the sites of mineralization is not a significant proof of its involvement in the process of mineralization (Siffert, 1950; Strates et al., 1971; Firschein and Urist, 1971, Dixit, 1972, Francis et al., 1973). Others have suggested a direct role for this enzyme in mineralization of the bone (Ali et al., 1971; Anderson, 1976, Bernard, 1978; Bab et al., 1979)..

Since discovery of matrix vesicles at the site of initial mineralization (Anderson, 1967, Bonucci, 1967) and the presence of alkaline phosphatase in these vesicles, the consensus has evolved that alkaline phosphatase is involved in the process of mineralization (Anderson, 1976). "Alkaline phosphatase brings about the hydrolysis of ester phosphates and thus raises the concentration of phosphates in matrix vesicles where Ca^{++} has already concentrated as a result of Ca^{++} -lipid interaction or active transport of Ca^{++} into matrix vesicles" (Anderson, 1976).

(ii) Ca^{++} , Mg^{++} -ATPase

ATPase is an enzyme which hydrolyzes Adenosine triphosphate into adenosine diphosphate and one molecule of orthophosphate.

action.

ATPases activated by divalent ions (Ca^{+2} , Mg^{+2}) appear to be important in calcium transport in a number of tissues. Nakamaru et al., (1967) showed a Ca^{++} activated ATPase in brain microsomes, which is related to calcium transport. Similar findings were reported by Shami and Radde (1971) in guinea pig placenta. Calcification or mineralization presumably also requires the active transport of calcium, with ATP as a possible source of energy for the "Calcium pump" (Ali et al., 1970; Anderson, 1973). Many earlier investigators have demonstrated ATPases activity in cartilage and bone although the enzyme was not well characterized (Ali et al., 1970; Rodan and Bourret, 1972). There have been indications that bone Ca^{++} , Mg^{++} -ATPase is actually not a separate enzyme but it is the same alkaline phosphatase that also possesses the ATPases activity (Felix and Fleisch, 1974, Felix and Fleisch, 1976). However, Skillen and Rahbani-Nobar (1980) have separated and characterised the alkaline phosphatase and ATPases and concluded that these two enzymes have different properties and ATPases is dependent upon Ca^{++} and Mg^{++} . Similar findings have been reported in odontoblasts (Granstrom et al., 1977), in bone (Bab et al., 1979); and in intestinal brush border (Sheila and Lawson, 1978).

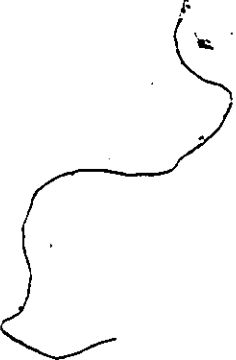
1.3.6 Acid Phosphatase: Its Role in Bone Metabolism

Acid phosphatase is a hydrolytic enzyme contained in

lysosomes and is often used as a marker to identify lysosome in histochemical studies (Dingle, 1977). It is present in a variety of cells including osteoprogenitor cells (Young, 1962), osteoblasts and osteocytes (Tonna, 1958, Vaes and Jacques, 1965; Warner, 1964, Susi et al., 1966; Jande and Grosso, 1975) and osteoclasts (Schajowicz and Cabrini, 1957). The presence of acid phosphatase in osteoclasts and their presence at the sites of bone resorption has been shown by earlier investigators (Burstone, 1960 a, b, Handelman, 1964; Susi et al., 1966). In a physiological study Doty et al., (1968) have shown that the level of acid phosphatase goes up in response to PTH injections with a concomitant increase in bone resorption.

In clinical conditions such as hyperparathyroidism and Paget's disease, which are accompanied by increased resorption, the activity of acid phosphatase has been shown to increase significantly (Klendshoj and Koepf, 1943; Gutman, 1952). Further it has been demonstrated that acid phosphatase activity also increases in the laying hen during the period of intensive osteoclastic medullary bone resorption which accompanies the formation of the eggshell (Taylor et al., 1965). In a review Vaes (1969) has implicated osteoclastic acid phosphatase in the process of bone resorption with the help of other lysosomal enzymes.

The presence of acid phosphatase in cells involved in production of bone matrix (osteoblasts) indicates that the enzyme



may be involved in that process. Its presence has been shown in the distended ends of Golgi saccules and elongated vesicles (Porter, 1964; Weinstock and Leblond, 1974; Jande and Grosso, 1975) in alignment with filaments. The various steps in collagen synthesis are well documented (Grant and Prockop, 1972). After hydroxylation of proline and lysine residues in ER the α -chains of protocollagen are transferred to Golgi complex (Revel and Hay, 1963; Porter, 1964, Weinstock and Leblond, 1974; Olson and Prockop, 1974). Before this material is secreted, a substitution of some of the hydroxy-lysine residues takes place with galactose or glucosyl galactose (Grant and Prockop, 1972). It has been shown that carbohydrate material in association with filamentous material containing labelled proline, occurs for the first time in these Golgi vesicles (Weinstock and Leblond, 1972). On this basis it has been suggested that the substitution occurs in the Golgi complex. It has also been shown that the synthesis of carbohydrate components of the glycosaminoglycans, a part of the extracellular matrix, occurs in the Golgi complex, where they are combined with the proteinic component and then secreted (Weinstock, 1972). The acid phosphatase, occurs in vesicles containing filamentous material and is present concomitant with the alignment of filaments (Jande and Grosso, 1975). The function of this hydrolytic enzyme is not apparent. Aminopeptidase, the enzyme related to the removal of the two ends of the protocollagen

molecule leading to the formation of the tropocollagen molecule, has been shown to be present in the intercellular matrix. The presence of acid phosphatase, as a marker, in the Golgi vacuoles, together with the filaments of protocollagen suggests the presence of aminopeptidase in these Golgi vacuoles. Thus, although the exact role of acid phosphatase is difficult to surmise the role for lysosome in bone collagen synthesis may be as stated above.

From the above discussion, it seems that the acid phosphatase in osteoclasts helps to resorb bone while that of the osteoblasts somehow plays a hitherto unknown role in bone matrix formation.

1.4 PERIODONTAL LIGAMENT

1.4.1 Histology

The Periodontal Ligament is a dense connective tissue that surrounds the tooth root. It connects the latter with the alveolar bone of the jaw. It is continuous with the connective tissue of gingiva and communicates with the marrow spaces through vascular channels in the alveolar bone.

The principal component of the periodontal ligament are collagen arranged in well defined bundles. The terminal fibres inserting into bone and cementum are known as Sharpey's fibres. The fibroblasts are the major cellular component of the periodontal ligament although other types of cells e.g. osteoblasts, osteoclasts, cementoblasts and epithelial rests of Mallassez, are also present.

1.4.2 Regional Differentiation in Periodontal Ligament

Depending upon the function and the position, the collagen fibres are divided into various groups as follows:

Transseptal group -

These extend interproximally over the alveolar crest and are embedded in cementum of adjacent teeth.

Alveolar Crest group -

These fibres extend obliquely from the cementum just beneath the epithelial attachment to the alveolar crest. Their function is to counter balance the coronal thrust of the more apical fibres, thus helping to retain the tooth within its socket.

Horizontal group -

These fibres extend at right angles to the long axis of the tooth from the cementum to the alveolar bone. Their function is similar to the one of the alveolar crest group.

Oblique group -

This is the largest group extending obliquely from cementum and attaching to the bone more coronally than their origin. They are the main stress bearers of the masticatory forces. (Provenza, 1972).

Apical group -

These fibres radiate from the cementum to the bone at the fundus of the socket and are absent in incomplete roots.

There are some elastic fibres and a few acid resistant oxytalan fibres. (Glickman, 1972).

1.5 MORPHOMETRIC TECHNIQUES FOR STUDIES OF SKELETAL TISSUE

1.5.1 Simple Measurements

An assessment of gross changes in bones, under experimental or pathological conditions, at organ level can be obtained by measuring the lengths and weights of the bones. These parameters may not provide indepth information but are a good starting point before deciding to investigate an experimental situation in detail (Garn et al., 1973).

A simple method to determine the inorganic contents of the bone is bone ashing. This technique can provide the valuable information on any alteration in mineral content of bones under any experimental or pathological condition.

1.5.2 Microradiography

The technique is as old as the discovery of x-rays. The technique was first applied to biological tissues by Goby (1913) and Lamarque (1936). Engström (1946) was first to apply this technique in bone investigations. Cohn and Lacroix (1953) made a comparison between microradiograms and ordinary biological preparations of the same samples and concluded that there was an inverse relationship between the degree of mineralization and the organic matrix. Densitometry of microradiographs allows a relative measurement of calcium deposits (Ponlot, 1960; Belanger et al., 1963). Microradiographs are like accurate maps of the relative calcium content of the various parts of a bone section at the histological level (Lacroix, 1972).

1.5.3 Topographic Mapping and Rates of Matrix Production

The concept of tracing skeletal and dental bone growth development, remodelling, and repair and of determining rates of matrix production is not new (Dixon, 1961; Frost, 1959, 1961, Harcourt et al., 1962, Vanderhoeft et al. 1962; Suzuki and Mathews, 1966, Tapp, 1966. Hong et al., 1968, Findlay and Steel, 1969; Soni, 1969, 1970; Soni and Messers, 1970, 1971, Sullivan, 1972; Cleall, 1974; Yen and Shaw, 1974). Numerous methods involving Alizarin red S, tetracycline, various radiotracers including calcium, strontium, and ^3H -labelled amino acids have also been applied in single or multiple doses, towards measuring various bone parameters.

1.5.4 Radioactive Tracers for Organic Components of Bone Matrix

The use of labelled amino acids to study the bone matrix formation is based upon the fact that these amino acids, which are precursors of certain matrical elements, are incorporated by the bone cells (osteoblasts and osteocytes) into macromolecules, which are secreted and become part of various components of the bone matrix (Leblond and Weinstock, 1972). The organic matrix makes about 30% of the total mass of the bone, 90% of which is the fibrous protein collagen (Eastoe and Eastoe, 1954; Eastoe, 1956). The collagen is very rich in the amino acids, glycine and proline. The proline is incorporated into the bone matrix via its hydroxylation to hydroxyproline which is found exclusively in animal tissues (Undenfriend, 1966). This makes

it a suitable precursor marker to study the formation of the bone matrix. Numerous investigators have used ^3H -glycine and ^3H -proline in quantitative autoradiographic studies of the bone matrix (Carneiro and Leblond, 1959; Tonna, 1965). In a series of comparative studies (Nirschein, 1967 a, b, 1969) it has been shown that ^3H -proline is a more specific label, for studying the bone matrix formation, than ^3H -glycine. In autoradiographic preparations the label appears in the form of reduced silver grains overlying newly formed bony surfaces indicating the location of the label. Properly time-spaced repeated administration of ^3H -proline, results in the formation of a topographic series of indelible bands from which the rates of skeletal growth and remodelling can be assessed (Tonna, 1974; Tonna, 1975). An alternative quantitative method is to measure the radioactivity in bone matrix or collagen by β -counters.

1.5.5 Radioactive Tracers for Inorganic Component of Bone Matrix

(i) Bone seeking elements

Certain elements when administered or ingested localize preferentially and specifically in the skeleton and are known as bone seekers. These include agents like alizarin and tetracycline which make complexes with calcium. Other elements included as bone seekers are those which either replace calcium or are present in normal bone in trace amounts. Of these, the major ones are Sr, Ra, Be and Ba. These are known as alkaline earths. The radioisotopes of these divalent cations have gained importance because of their use in the quantitative studies of bone

matrix and mineralization and because of their hazards due to retention of isotopes in skeleton. Other elements Na, K, Mg which are present in bone are rarely used in bone investigations (McLean and Budy, 1964).

As bone matrix is deposited, the mineral ions, calcium and phosphate also appear in the matrix. The details of the process of calcification will not be dealt with here as these are discussed in another section (page 13). Early studies (Leblond et al., 1950) showed that when ^{32}P or ^{45}Ca is injected into newborn rats or mice the radiographic reaction is uniformly distributed throughout the whole substance of the bone. However, Lacroix (1953) and Leblond et al. (1959) later showed that after injection of ^{45}Ca only growing osteons show a significant uptake of radioactivity. Radioisotopes of Strontium (^{85}Sr , ^{90}Sr) also behave like Ca and have been used as markers of calcification of bone matrix (Harrison et al., 1967).

Fluoresceins

The use of fluoresceins as fluorochromic labels for bone and dental tissue growth, remodelling, and resorption has received attention, particularly their combined use with other fluorescent markers to produce polychromatic banding (Suzuki and Matthews, 1966; Modis et al., 1969; Rahn and Perren, 1970; Hammond and Storey, 1974; Soni and Malloy, 1976).

Calcein, a fluorescein - iminodiacetic acid complex,

fluoresces green when combined with calcium (Diehl and Ellingboe, 1956). It has been used in bone studies of various species (Modis et al., 1969; Sullivan, 1972; Hammond and Storey, 1974; Soni and Malloy, 1976).

The alizarin red S and fluoresceins do not provide any advantages over tetracycline but they do help in getting polychromatic banding pattern. The rates of bone matrix formation can be calculated by dividing the distance between the two bands by time.

(iii) Tetracycline

The antibiotic tetracyclines make complexes with a great number of metal ions such as Fe^{+3} , Cu^{+2} , Ni^{+2} , Ca^{+2} , Fe^{+2} , Co^{+2} , Zn^{+2} , Mn^{+2} (Albert, 1953, Albert and Rees, 1956, Conner, 1956; Higuchi and Bolton, 1959; Ibsen and Urist, 1962; Urist et al., 1962) and then fluoresce under the UV light. Oxytetracycline complexes with Ca are much stronger as compared to its complexes with organic compounds (Ibsen and Urist, 1964). More tetracycline is incorporated into newly forming bone as compared to older bone because of greater chemical reactivity, high vascularity, smaller crystal size, more hydration and looser packing of organic and inorganic constituents of matrix in the new bone (Ibsen and Urist, 1964). Many investigators have taken advantage of this property of tetracycline and have applied it for studying the various parameters related to bone mineralization (Milch et al., 1958,

Harris et al., 1962, Owen, 1962, Frost and Vallaneuva, 1960; Berquist, 1962; Berquist and Hulth, 1963). Properly time spaced multiple administration of tetracycline results in the formation of fluorescent labels (in UV) from which the rates of bone growth and resorption can be measured (Baylink et al., 1970). It can either be used as a label and the distances between various labels can be measured to see the total bone formation or its incorporation can be quantitatively assessed and results used as quantitative measure of the mineralization. The high affinity of tetracycline for Ca^{+2} makes it a very suitable marker for studying the initial phase of the mineralization (Lacroix, 1971). Another advantage over Isotopic calcium is that tetracycline can be visualized under UV light and quantitative measurements can be made with cold tetracycline (Kelly and Buyske, 1960; Cohen, 1962; Ibsen et al., 1963).

(iv) Alizarin

Alizarin is one of the oldest bone markers. In histology a soluble salt, alizarin red S, is used for the demonstration of calcium. The structures of alizarin and tetracycline are quite similar. In general, their distribution in the skeleton is also similar, as is their interaction with hydroxyapatite (Harris et al., 1962; Myers, 1968). Alizarin red S fluoresces under UV light and administered in low doses serves well as an intravital fluorochrome (Adkins, 1965). Alizarin red S has been used in combination with other fluorochromes in multiple dose experiments

to produce polychromatic banding (Modis et al., 1969; Cleall, 1974; Hammond and Storey, 1974).

(V) In VIVO Estimation of Bone Mineral by Photon

Absoptiometry

The technique is based on the principle that the mass of mineral existing in a bone is directly proportional to the amount of photon energy absorbed by the bone (Cameron and Sorenson, 1963). The scanner module provides a collimated beam of monoenergetic photons from a sealed source. The source is synchronized with a scintillation detector and both traverse the bone simultaneously. Cameron and Sorenson (1963), have developed this technique for in VIVO measurement of bone mass and have used it to evaluate the mineral content of bone and width of the bone (Sorenson and Cameron, 1967, Cameron et al.; 1968, Jette, 1973; Boyd, 1973). The roentogenic methods though widely used, are not considered sufficiently precise to detect changes in mineralization in experimental and pathological conditions where the differences are too minute (Lachmann, 1955).

1.5.6. OBJECTIVES AND RATIONALE BEHIND THE INVESTIGATIONS

Since the previous studies about the effects of the lathyrogens on hard tissues, as discussed earlier, reported only gross changes in skeletal structures and were mainly related to teratogenic effects of lathyrogens, an investigation to look into the precise changes in bone which would provide detailed information about the exact parameters in the bone metabolism affected by B-APN, was considered desirable.


Due to the known effects of the lathyrogen B-APN on collagen fibril maturation, the collagen being a major component of bone matrix, it was considered important to evaluate morphologically the effects of B-APN on collagen fibrils and collagen synthesizing cells. The Periodontal Ligament (PDL) is ideally suited for such a study as collagen fibrils are specifically oriented and unlike in bone are free from any mineral deposits.

The role of collagen fibrils in the mineralization of the bone matrix has been considered of great significance in the past (as discussed earlier). The inter- and intra-molecular cross linkages were considered a pre-requisite for collagen fibrils to become mineralized (Mills and Bavetta, 1968) and inhibition of these links by B-APN came to be believed as the mechanism of B-APN action in producing the various osteopathologies (Barrow et al., 1974). Based on the fact that inter- and intra-molecular cross linkages are inhibited by B-APN, Rosenquist


et al., (1977) conducted a morphological study and documented that the initial rate of mineralization of the bone matrix was inhibited. Our studies, morphometric and radioisotopic using ³H-proline and ³H-tetracycline incorporation, not only confirmed but improved the data on quantitative evaluation of the B-APN effects on the initial mineralization in rats and chicks. The rationale of using chicks after our study in rats has been discussed in the appropriate section.

Rosenquist et al., (1977) speculated that the cross linkages inhibition by B-APN might not be the mechanism of its action on mineralization as a similar cross linkages inhibition in Vitamin D deficiency does not result in the inhibition of rate of initial mineralization. In recent years the emphasis on collagen fibrils as the nidation site for the initial mineralization has shifted to the matrix vesicles in bone and cartilage and these have emerged to be a more likely site for initial mineral deposits. Furthermore, there are reports of presence of certain enzymes, believed to be involved in the process of mineralization, on matrix vesicles membranes (as discussed elsewhere in the thesis). In view of the absence of any convincing mechanism of B-APN action on the mineralization, an investigation into matrix vesicles and enzymes related to the mineralization were considered important.

The ultrastructural and biochemical studies on bone are



hindered by the difficulty in preparing the bone tissue for various studies. The embryonic bones provide a suitable material to study all the parameters of bone metabolism. The development of bones in chick embryo have been studied extensively and various stages are well documented (Kell, 1925). Because of these reasons the last phase of this investigation was conducted on the chick embryonic bones.



CHAPTER 2.

MATERIALS AND METHODS

2.1. ANIMALS

Studies on the Rats

For the study of the periodontal ligament and that of the bone matrix formation and its mineralization in rats, four week old male Sprague Dawley rats weighing about 69 ± 2 gms were purchased from Bio-Breeding, Ottawa Ontario, Canada.

All rats were fed on a powdered Ralston Purina Rat Chow ad libidum. B-APN in powder form was mixed with the feed for the experimental rats. Total B-APN concentration in the food was 0.25 percent. No data on the food consumption was considered essential because no differences in food consumption between controls and the experimental animals was very similar.

Studies on Chicks

For the study of the effects of B-APN on the bone matrix formation and its mineralization two week old white leg horn chicks weighing 200 ± 10 gms were used.

All chicks were fed a powdered Biostartena diet (Purina Chow Company, Whitby, Ontario) ad libidum. However, the experimental chick feed was supplemented with B-APN. B-APN concentration in the feed was 0.05 percent.

2.2 LIGHT MICROSCOPY

(i) Periodontal Ligament-

For the histology of the periodontal ligament, rats were perfused after anaesthetization with intraperitoneal injections of the chloral hydrate (35mg/100gm body weight), with a fixative through the ascending aorta. The perfusion fixative contained 1% glutaraldehyde in 0.09 M phosphate buffer at pH 7.4. This is a slight modification from Sotelo and Palay, (1967) to adjust the osmolarity of the fixative at 350 milliosmoles. After 20 minutes of continuous perfusion, the lower jaws were further fixed in $\frac{1}{2}$ strength Karnovsky's fixative (Karnovsky, 1965) for 24 hours.

The jaw bones were decalcified with 4.13% EDTA pH 7.4 at 0-4°C for 3 weeks with weekly changes and under constant gentle stirring. The decalcifying solution also contained 2% glutaraldehyde.

After decalcification the bones were sliced buccolingually, each slice about 2mm. thick and washed with phosphate buffer (0.1M, pH 7.4) containing 7% sucrose for 2 hours with 3 changes.

For light microscopy some pieces of bone were then dehydrated through a graded sequence of ethanol and xylene and embedded in paraffin wax. 8 μ m thick sections were stained with Masson's trichrome staining technique.

2.3 Electronmicroscopy

(i) Periodontal Ligament

The slices of bone obtained as described above were further post-fixed in 1% OsO₄ in 0.1M phosphate buffer (pH 7.4) for 2 hours (0-4°C), and dehydrated with ethanol. Araldite was used as an embedding material. The ultrathin sections were cut on a LKB ultramicrotome and picked up on copper grids. The sections were stained in sequence with 1% aqueous phosphotungstic acid, 2% uranyl acetate in 50% ethanol and aqueous lead citrate (Reynolds, 1963).

(ii) Chick embryonic Tibia

For electronmicroscopy of the embryonic chick bones the tibias were fixed with 2.5% Glutaraldehyde in cacodylate buffer (0.1M, pH 7.4) for one hour at 0-4°C. The bone pieces were further sliced to a thickness of 60-100 μ m with a Sorval tissue cutter. These sections were washed in 0.1M cacodylate buffer containing 0.2M sucrose for 1 hour with 2 changes. The undemineralized pieces were post fixed with 1% OsO₄ in cacodylate buffer for 1 hour. The dehydration was done in graded series of alkaline alcohols. For decalcification of sections where required, 4.13% EDTA with 2% Glutaraldehyde (pH 7.4) was used. Araldite was used as embedding medium. During ultrathin sectioning, all the precautions described by Boothroyd (1964) were taken to prevent the loss of minerals.

2.4 HISTOCHEMISTRY

For enzyme localization the bones were fixed in cacodylate buffered 2.5% Glutaraldehyde for 1 hour at 0°C (Sabatini et al., 1963, Matsuzawa and Anderson, 1971). From the diaphyseal centre of each tibia 50 μ m thick sections were cut with the Sorvall Tissue Chopper. These sections were decalcified in 4.13% EDTA containing 2% Glutaraldehyde, buffered with cacodylate buffer at pH 7.4 for 2-3 hours. After decalcification the sections were rinsed in 0.1M cacodylate buffer containing 0.2M sucrose (pH 7.4) thrice for 10 minutes.

A. ALKALINE PHOSPHATASE

(i) Light Microscopic Localization

At light microscopic level the alkaline phosphatase histochemistry was done according to a technique described by Burstone, (1958). Twenty μ m thick sections from embryonic tibias were cut with the help of a freezing microtome. These sections were floated for 1 hour in naphthol AS-BI phosphate diazonium salt medium (a filtered solution of 2.5mg naphthol AS-BI phosphate dissolved in 1 ml N, N-dimethyl formide to which was added 50 ml 0.05M tris buffer (pH 8.3) and 40 mg Fast Red Violet LB). As a control for histochemical procedure small pieces of rat kidney were incubated in the same vials as the pieces of embryonic bones. The sections were rinsed several times with tris buffer and were floated on to glass slides and were mounted with glycerine jelly. The alkaline phosphatase

activity was seen as a vivid red azo dye deposit.

(ii) Electronmicroscopic Localization

After decalcification, as described above, 50 μ m thick sections from the diaphyseal centre of each embryonic tibia were cut with the help of a tissue chopper. These sections were incubated in special media for 1 hour at 37°C. As a control for the histochemical procedure small pieces of rat kidney were incubated for demonstration of alkaline phosphatase in the same incubating vials as the pieces of embryonic bones. After incubation, the tissues were rinsed in cacodylate buffer twice for 10 minutes. Some sections were treated with ammonium sulfide (0.1%) for 5 minutes and these were used for light microscopic evaluation of the reaction.

For electronmicroscopy, the sections were postfixated in OsO_4 solution for 30 minutes, dehydrated in graded ethanols and prepared for electronmicroscopy as described earlier.

The constituents of the incubating medium which was prepared according to Bernard (1978) were as follows:

Tris maleate buffer, pH 8.6 (0.1M)	10 ml.
Sod-B-glycerophosphate, 1.25%	10 ml.
Double distilled water	23.5 ml.
Magnesium chloride 10%	3 drops
Lead nitrate 1%	6.5 ml.

All solutions were made with freshly boiled and cooled water which had been double distilled earlier.

Lead nitrate was added drop by drop while the solution was constantly stirred with a magnetic stirrer. Unless freshly boiled water was used, a precipitate developed on addition of nitrate. The preparation of the incubation medium was found to be very critical.

B. Acid Phosphatase

(i) Light Microscopy

For acid phosphatase histochemistry the technique used was that of Burstone, (1958). The fixation, decalcification and sectioning procedure were similar to as described for alkaline phosphatase earlier. Similarly prepared rat kidney sections were kept as control for histochemical procedure.

The free floating sections were incubated for 30 minutes in an incubating medium which was prepared from three stock solutions.

The constituents of three stock solutions were as follows:

Stock Solution A:

Sodium α -naphthyl acid phosphate (4 mg/ml) was dissolved in Michaelis veronal acetate buffer stock solution (9.714 gm sodium acetate, 3 H₂O + 14.714 gm sodium barbiturate in carbon dioxide distilled water, made up to volume of 500 ml.

Stock Solution B.

2 gm pararosanilin-HCl was added to 50 ml 2N HCl and was gently heated. After cooling the solution was filtered once.

Stock Solution C.

4% Sodium Nitrite in distilled water.

The incubating medium was constituted as described below:

- 1- 5 ml of solution A was added to 13 ml distilled water
- 2- 0.8 ml of B and C each were mixed together and were then added to solution 1.
- 3- pH was adjusted to 6.5 with 1N sodium hydroxide and the solution was filtered.

The sections, after incubation, were mounted in glycerine jelly.

(ii) Electronmicroscopy

For electronmicroscopic localization of acid phosphatase the fixation, decalcification, sectioning and processing was done according to a schedule already described for alkaline phosphatase.

The sections were incubated in special medium for 20 minutes. The incubating medium was constituted as follows:

Tris maleate buffer (pH 5.2, 0.1M)	10 ml.
Sod-B-glycerophosphate 1.25%	10 ml.
Double distilled water	10 ml.
Lead nitrate 0.2%	20 ml.

All solution were made from freshly boiled cold water. Lead nitrate solution was added drop by drop while the solution was constantly stirred with a magnetic stirrer. A clear solution was obtained and centrifuged for 10 minutes.

2.5. MICORADIOGRAPHY

(i) Preparation of Bone Sections

The bones were fixed in 70% ethanol for 24 hours. These were then cut at the desired sites with a diamond dental burr and dehydrated progressively in 95% ethanol, in three changes of absolute ethanol the following day, and four changes of acetone every two hours thereafter.

The bone pieces were then infiltrated with increasing concentration of Epon resin. The Epon resin mix was made according to schedule described by Luft (1961) and summarized below:

Solution A: 62 ml Epon 812

100 ml DDSA (Dodecenyl succinic anhydride).

Solution B: 100 ml Epon 912

89 ml MMA (Nadic methyl anhydride).

The infiltration method is based on the schedule described by Belanger (1963). The infiltration mixtures were made as following:

1. Mixture 1: Solution A: Solution B: Acetone

1:1:6

2. Mixture 2: Solution A: Solution B: Acetone

1:1:2

3. Mixture 3: Solution A: Solution B: Acetone

3:3:2

To this was added 1.5% (v/v) of DMP accelerator (2, 4, 6-trimethyl amino ethyl phenol).

4. Mixture 4: Solution A: Solution B

1:1 and 1.5% (v/v) of DMP was added to it.

During the first hour of the infiltration schedule, the bones in the test tube containing mixture 1 were centrifuged at 1800 R.P.M. and the stoppered tubes were stored in a dessicator overnight. Next day the bones were again centrifuged for two hours with mixture 2, followed by another two hours in mixture 3. Finally, the bones were infiltrated under vacuum (20 mm Hg) for 3-4 hours with mixture 4. Each bone sample was then embedded in a plastic mold partially filled with fresh mixture 4 and the molds were stored for thirty minutes under vacuum at room temperature. Fresh resin was added to fill the molds. The Epon resin was allowed to polymerize in an oven at 35°, 45° and 60°C for 24 hours each.

After Epon polymerization, the blocks were trimmed with a bandsaw and cemented on to a thin plate of polyglass fitting the cutting table of the Gilling diamond-wheel cutting machine (Gilling and Bounocore, 1959), sections were further ground and polished with the help of very fine grate sand paper and were further washed in zephran chloride (1:700). Final thickness of sections was 100 ± 10 μ m. The sections were picked up by a strip of scotch tape and were affixed to a glass slide so that during storage each section was sandwiched between the tape and the slide.

The spectroscopic slides used for recording of the x-ray

images of the bones were obtained from Kodak of Canada, Toronto. These slides are coated with emulsion EK 649.0. These were exposed to x-rays in a specially designed microradiograph unit built by Garth WestenKow Co. and the Jackson Scientific Machine Co. of Salt Lake City Utah.

Before use, the spectroscopic slides were coated with a thin layer of celloidin by dipping them in a 1% solution of celloidin in ethanol-ether, and were dried for twenty-four hours.

In the dark room, the bone sections adhering to the tape were lifted from the storage glass slides and transferred to the emulsion covered plate so that the bone section now was sandwiched between the tape and the photographic emulsion. Intimate contact between bone and emulsion was essential to ensure high resolution.

(ii) Exposure

The microradiograph unit consists of a high voltage power supply, a line voltage regulator, a control panel, x-ray tube and a camera to position the film and the specimen to be x-rayed. The emulsion slide and sections apposed to it were placed in a specially designed camera in a dark room. The camera tube was placed on to the outlet for x-rays. The camera was rotated to bring the section in the path of x-rays. The sections were exposed to x-rays generated at a regulator voltage of 115 volts, an x-rays voltage of 10,000 volts and an x-ray

current of 10 milliamperes. The time of exposure varied for different sets of section. For cross sections of tibial diaphyses a 10 minute exposure time was found to give proper exposure. All other conditions remained constant.

The exposed emulsion slides were removed from the camera in dark room. These slides were treated with acetone to remove the protective covering of celloidin. The photographic plate was then developed for 10 minutes in Eastman Kodak D-19 developer at 20°C and then fixed for 15 minutes in Kodak acid fixer with hardner. Slides were washed for 30 minutes, air dried and mounted with a coverslip with Permount.

2.6 FLUORESCENT MICROSCOPY

For fluorescent microscopy the tetracycline was used as a fluorescent marker. The rats and chicks used in these experiments were injected with tetracycline I/P (30 mg/kg body weight). The tetracycline was obtained as tetracycline hydrochloride from Pfizer Co. Ltd., Montreal, Canada. The bones were embedded in Epon 812 according to the method and schedule described for microradiography on page 45. Fifty μm sections, from approximately similar regions of the bones, were cut with a diamond saw (Thin sectioning machine, Harco Machine, Inc., Rochester, N.Y.). These sections were further ground to a thickness of 25 μm with the help of very fine grate sand paper under running water. The sections were then washed with Zephran Chloride (1:700), double distilled water and dehydrated with

rising concentration of alcohols and xylene. These sections were mounted on glass slides with the help of a nonfluorescent mounting medium and were examined under UV light. From each section the measurements were taken from at least 5 regions and at least 5 sections from different rats and chicks were examined. The width between two labels was taken as the linear bone apposition between two intervals of time.

2.7 SERUM CALCIUM ESTIMATION

The serum Ca^{++} was estimated with an automatic calcium analyzer (940 calcium analyzer, Corning Scientific, Medfield, Mass., U.S.A.). It is known that the dye calcein, a fluorescein derivative, forms an intensely fluorescent, non-dissociated complex with calcium ions in an alkaline medium. The analytical procedure incorporated in the Model 940 is based upon the quenching of this fluorescence by chelating the calcium ions with the titrant EGTA.

Reagents used for serum Ca^{+2} determination are:

- Potassium hydroxide (KOH) IN
- Ethylene glycol bis (B-amino ethyl ether) N, N'-Tetraacetic acid. 0.04% wt./vol. solution.
- Calcium standard 10.00 mg% or (5.00 m Eq/liter).
- Calcein indicator dissolved in calcium standard.

Procedure

- Cuvette is filled up to the mark with KOH.
- 100 μl of calcein indicator was added and instrument was left

running for 10 minutes.

- The instrument was calibrated with calcium standard. Three consecutive readings not differing by more than 0.05 mg% was taken as calibration point.
- 100 ul of test serum was added and titrated.
- The readings were made directly in the digital meter of the calcium analyzer.

2.8 BIOCHEMICAL TECHNIQUES

(A) Liquid Scintillation -

The scintillation counting technique is based on the interaction of nuclear radiation with matter. In the scintillation counters use is made of the photons created when a suitable luminescent material, the scintillation cocktail, is excited by nuclear radiations. These photons are collected at the cathode of a photocell which converts them into electrons. The multiplication of these electrons in the photomultiplier will give rise to electrical pulses which can be recorded. The liquid scintillators can be used for counting of weak beta emitters.

For our experiments the radio isotopes (^3H -proline as L-(2, 3- ^3H) - proline and ^3H -tetracycline as (7- ^3H (N)-tetracycline), were obtained from New England Nuclear Corporation of Canada. Lachine, Quebec.

Bone tissues for scintillation counting were at first cleaned of soft tissues, demarrowed and weighed. Each bone was

then dissolved in 2 ml 6N HCl overnight at 80°C. Five 100 ul aliquots were taken from each sample. Aquasol was used as a scintillation cocktail. The NCS (NEN Canada, Lachine) was used as a tissue solubilizer. The radioactivity was counted with Beckman 100 scintillation counter. The incorporation of isotopes were calculated as a percentage of the initial dose administered per unit weight of bone.

(B) Enzymes -

The activities of alkaline phosphatase, Ca^{++} -ATPase, and Na^{+} - K^{+} , ATPase were evaluated not in the whole bone homogenates but in homogenates of membranes of the cells obtained from the Bone.

(i) Isolation of Membrane and Subcellular Fractions from Chick

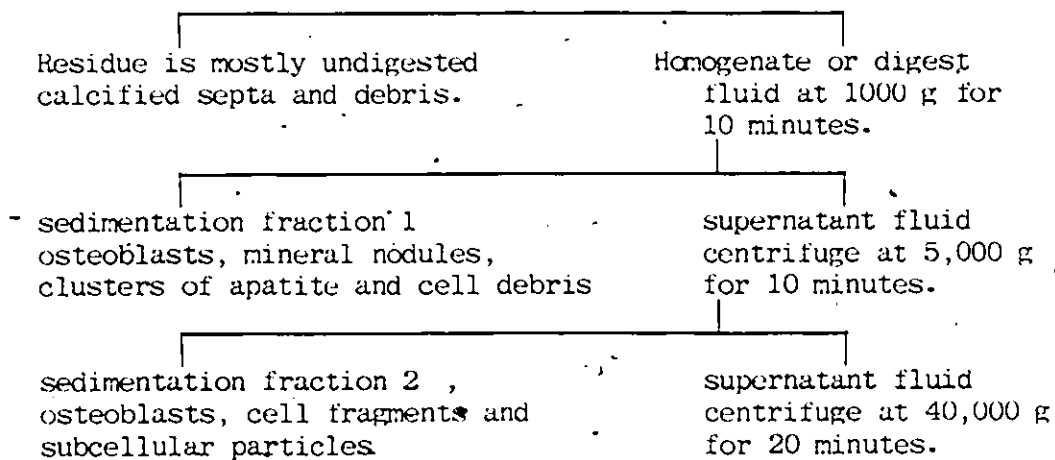
Embryonic Bones -

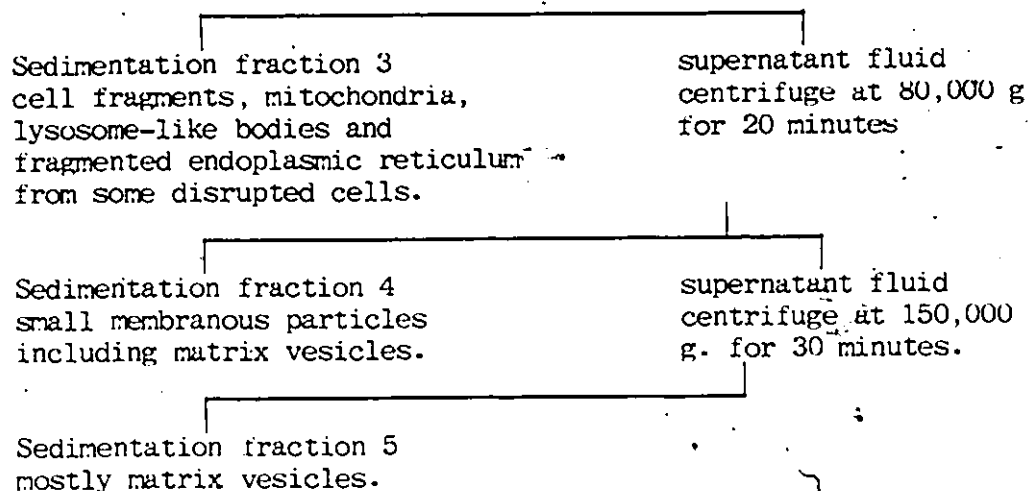
The embryos were sacrificed and their tibias were removed. All the following procedure was performed at 0-4°C and under aseptic conditions. The bones were exposed by removing all the superficial tissue from bone with a sterile forcep. The bone pieces were further minced into small fragments and were homogenised for 1 minute by a motor driven Teflon Pestle in 50 mM tris (pH 7.6) containing 3.75 mM mercaptoethanol. The collagenase solution (5 ml/g wet weight of bone) was added to the bone samples and was incubated at 37°C in a shaker for 4 hours.

The collagenase solution, required for digestion of bone

was prepared according to the method described by Ali (1979). Sigma (No. C-0130) crude collagenase type I was prepared to contain 1000 units/ml in N-tris (Hydroxy methyl) - 2 aminoethanesulfonic acid (TES) - sucrose buffer. Sucrose solution (0.25M) was made upto volume in 50 mM TES buffer, adjusted to pH 7.4 with 1N NaOH. Gentamycine was included to contain 5 mg/100ml of solution. The collagenase was allowed to dissolve in the TES-sucrose solution for 10 minutes at 37°C, and this fluid was cleared by centrifugation in a Beckman centrifuge at 150,000g for 130 minutes. The brown supernatant collagenase solution was passed through millipore filter No. AP 2502500 and was sterilized by filtering through Millipore filter No. GWSPO 2500 with a 0.22 μ m pore size. The collagenase solution was prepared in bulk and was stored at -20°C in 10 ml aliquots in sterile bottles.

After digestion, the homogenate (fraction 0) was centrifuged according to the following scheme described by Ali, (1979).





The activity of enzyme alkaline phosphatase, Ca^{++} -ATPase, Na^+ , K^+ -ATPase was estimated in fraction 5 while acid phosphatase activity was determined in fraction 2.

(ii) ENZYMATIC ASSAYS

(a) Acid Phosphatase

The organic phosphate esters are hydrolysed by acid phosphatase. In this method the enzyme is allowed to hydrolyze an organic phosphate and the activity is measured as the concentration of the free organic part of the substrate.

0.25 ml of the sample was mixed with 1.65 ml of 0.1M tris maleate buffer, pH 5.2 for 10 minutes at 37°C to destroy glucose-6-phosphate. The reaction was started by the addition of 0.1 ml of substrate solution, giving a final concentration of 8 mM 4-nitrophenyl phosphate. After 30 minutes of incubation the reaction was stopped with 2 ml of ice-cold tris-phosphate reagent and read at 400 nm immediately in an spectronic 20 spectrophotometer.

A standard stock solution was made by adding 0.1 ml of 10 mM/ml p-nitrophenol into a 10 ml flask containing 0.1 M tris maleate buffer pH 5.2.

Substrate Solution -

160 mM 4-nitrophenyl phosphate disodium salt. The tetrahydrate (mol. wt. 335.1) is dissolved at 0.119g/2ml.

Trisphosphate reagent -

1M Tris-HCl, pH 8.5, containing 0.4M K_2HPO_4 .

Tris 12.1 g.

Water 60 ml.

K_2HPO_4 5.43 g.

Adjust to pH 8.5 with HCl and make up to 100 ml with water.

(b) Alkaline Phosphatase

The principle for alkaline phosphatase estimation is similar to one described for acid phosphatase.

0.1 ml of the sample was mixed with 0.75 ml of tris maleate buffer (71.43mM, pH 9.3) for 10 minutes at 37°C for the same reasons as described for acid phosphatase. The reaction was started by adding 0.1 ml of substrate solution, which gave final concentration of 5mM nitrophenyl phosphate. After 30 minutes of incubation the reaction was stopped by adding 3 ml of 0.1 N Na OH and read at 400 nm immediately in spectronic 20 spectrophotometer.

Reagents-

A standard stock solution was made by adding 0.2 ml of 10mM/ml p-nitrophenol into a 10 ml flask containing 50 mM tris maleate buffer pH 9. The final concentration will be 0.2 mM/ml.

Substrate solution -

A 50 mM solution was made by dissolving 0.185 g p-nitrophenyl disodium salt per 100 ml double distilled water.

Ions -

Mg Cl ₂	50 mM	0.068 gm/200 ml.
Zn Cl ₂	2.5 mM	2.033 g/200 ml.

(c) Ca⁺⁺, Mg⁺⁺-ATPase

This test is based on the principle that the enzyme hydrolyzes the substrate ATP to ADP and Pi. Ammonium molybdate is added which binds the Pi released from ATP. To reduce the molybdate; Fiske-Subbarow reagent is added and Pi release is measured as a measure of ATP hydrolysis and the results are expressed as Pi released/mg. protein/hr.

0.1 ml of test sample was added to 0.7 ml of 71.43 mM tris HCl buffer (pH 7.4) and was preincubated for 10 minutes. To this was added 0.1 ml of substrate solution and 0.1 ml of ions and the mixture was incubated for 60 minutes. The reaction is

stopped by 2 ml of 10% trichloroacetic acid (TCA). After this 1 ml of ammonium molybdate solution was added and left for 10 minutes. To this was added 250 ul of Fiske-Subarow solution and read in a spectronic 20 spectrophotometer at 610 nm.

Reagents -

Standard stock solution -

KH_2PO_4 0.4 mM/l to obtain this dissolve 0.00544 gm/100 ml. in 0.05 M tris HCl (0.079 gm/100ml).

Substrate solution -

30 mM ATP (F. W 596.2) is made by dissolving 0.09 gm of ATP in few drops of D.D.W. and to this 14 drops of 1N NaOH is added and made to 5 ml with D.D.W.

Trichloroacetic acid (TCA) 10%

Ions CaCl_2 100 mM

MgCl_2 50 mM

Molybdate Solution - 1.25 gm ammonium molybdate dissolved in 100 ml of 2.5 N Sulfuric acid.

(d) Na^{++} , K^+ -ATPase

Principle and the procedure for Na^+ , K^+ -ATPase is similar to one described for Ca^{++} , Mg^{++} -ATPase. Here only the differences in ions and concentration of substrate solution will be mentioned.

Substrate solution -

6 mM ATP (F.W. 596.2) is made by dissolving 0.015 gm of ATP in few drops of D.D.W., 14 drops of 1N NaOH and made to 5 ml

with D.D.W.

Ions -

NaCl 100 mM

KCl 10 mM

(iii) Total Protein Estimation -

This analysis is based on the principle that proteins combine with Cu^{++} (used as Cu^{++} reagent) and form a copper-protein complex. When Folin phenol is added to this copper-protein complex, copper is released and the colour develops which is compared against the standard albumin dilutions.

0.9 ml of D.D.W. and 0.1 ml of test samples were mixed and 5 ml of Cu^{++} reagent was added and allowed to react for 30 minutes. The absorption and transmittance was read in a spectronic 20 spectrophotometer at 500 nm.

Reagents -

Cu^{++} Reagent - made in 100 ml

Na OH 0.1N

Na CO_3 2 gm/100 ml

Cu SO_4 1%

K^+ and Na^+ tartrate 1% each

Folin Phenol 2 N

Standard Albumin obtained from sigma chemicals as Bovine serum albumin fraction V dissolved to make 100 mgm/ml.

(iv) Bone Ash Determination

Chicks were sacrificed and their tibias were stored in gauze moistened in 10% buffered formalin solution, pH 7.4.

These bones were dissected free of soft tissue and sawed at each end to obtain diaphyseal bone (middle third) from which the bone marrow was removed by a jet of air. The diaphyses were then dehydrated and defatted in acetone, changed daily for 3 days. Subsequently the bones were air dried at room temperature, weighed, and then ashed at 600°C for 16 hours in an incinerator. The ashes were cooled for 2 hours and weighed. The method is similar to one used by Rosenquist et al., (1977).

2.9 Incubation of Eggs and preparation of embryo bones for various studies.

The eggs were obtained from the same hatchery throughout the experiments. They were of White Leghorn variety. The incubation of the eggs always started one day after arrival and at noon time.

The eggs were incubated in an incubator, built by Pèrsime Incubator Co., Gettysburg, Ohio, at 37°C and 58% humidity with automatic tilting every two hours.

On 7th day of incubation the eggs were candled and unfertilized eggs were discarded. The fertilized eggs were cleaned with a cotton swab soaked in absolute alcohol. A hole was made in each egg with a sharp instrument to open the air chamber. The dissolved drug was deposited on the inner shell membrane. After administration of the drug to one group, and control vehicle to the other, the holes were closed with a white paper tape. The eggs were placed back into the incubator. On the predetermined time the embryos were removed by cracking the egg shell and placed in a petri dish. The presence of the heart beat was taken as the criteria for marking the embryo alive. The membranes were removed and the embryos were weighed. For further studies the mineralized portions of their tibial diaphyses were removed under a dissecting microscope and prepared for various experiments as described in the preceding sections.

CHAPTER 3.

RESULTS

3.1 STUDIES ON THE PERIODONTAL LIGAMENT AND ALVEOLAR BONE OF RATS FED ON A DIET CONTAINING β -AMINOPROPIONITRILE

The rats fed a diet containing B-APN were generally lethargic and showed kyphosis of their spines.

The periodontal ligament (PDL) and alveolar bone of the rats, fed a diet containing B-APN (0.25%) for four weeks, were examined with microradiography and light microscopy. The periodontal ligament was further examined histochemically and with electronmicroscopy.

3.1.1 MICRORADIOGRAPHY

For microradiographic examination the interdental septae of the first and second molars as well as the inter radicular septae of the first molars were chosen. Figs. 3.1.1 and 3.1.2 are microradiographs of the hemi-mandibles of normal rats taken from the lingual and buccal sides respectively. Similarly Figs. 3.1.3 and 3.1.4 are from one hemi mandible of B-APN treated rats taken from similar directions as mentioned for the controls. These microradiographs were found to be very informative for evaluation of the gross changes in alveolar bone and the periodontal ligament space.

The microradiographs of the mandibles of the control rats showed the interdental septae, which were quite radio-opaque, had smooth outlines next to the periodontal ligaments and at their crests (Figs. 3.1.1,2). The tip of the interdental septae between the first and second molars extended up to the cemento-

enamel junction of the distal root of the first molar. The shape of the crest of these septae was conical (Fig. 3.1.1). The inter radicular septae of the first and second molars extended quite high in the bifurcation of the roots and were less radio-opaque than the interdental septae (Fig. 3.1.2).

A careful examination of the microradiographs of the rats treated with B-APN showed the interdental septae to be less radio-opaque than those in the controls (Fig. 3.1.3). The height of the interdental septae between the first and second molars were lower and the septae did not reach the cemento-enamel junction. Thus, some coronal part of the root was left without bony support (Fig. 3.1.4). The crests of these septae were obtuse and not conical as seen in the controls (Fig. 3.1.4). The inter radicular septae between the two roots of the first molar showed a lower radiodensity compared to the controls. The apical two thirds of the interdental and inter radicular septae were quite radiolucent (Fig. 3.1.4).

The microradiographs of the longitudinal section of the mandible from the controls confirmed the observations described above from microradiographs of the hemi-mandibles. The interdental and inter radicular septae presented an appearance which was quite regular and the nutritional spaces containing blood vessels etc., were also very regular (Fig. 3.1.5).

The microradiographs of the longitudinal sections of B-APN treated rats, through the interdental and inter radicular septae,

D

is shown in the Fig. 3.1.6. Both these septae were quite different from the controls. The borders of these two types of septae were quite irregular (Fig. 3.1.6). In treated material the nutritional spaces were quite large and often irregular. The large spaces from the borders of these septae seem to merge with alveolar bone medullary cavities. The apical third of each of these septae was less radio-opaque compared to the controls (Fig. 3.1.6). The amount of the bone material in these septae, even from visual examination of the microradiographs, was quite low compared to the controls. The width of periodontal ligament space appeared to be larger compared to that of the controls (Fig. 3.1.6).

3.1.2 LIGHT MICROSCOPY

Transseptal fibres, in the controls, were seen to run from cementum of each tooth directly towards the cementum of the adjacent tooth (Fig. 3.1.7). The fibroblasts were randomly arranged among the collagen fibres and were of fusiform shape. In B-APN fed rats the transseptal fibres (Fig. 3.1.5) were seen to run in a similar fashion and the arrangement and shape of the fibroblasts were also comparable to the controls.

The alveolar crest fibres extending from the cemento-enamel junction to alveolar crest were seen to be arranged in well defined bundles and the fibroblasts were arranged randomly among the collagen fibres (Fig. 3.1.9). The attachment of the collagen fibres to cementum and the alveolar crest was very

regular (Fig. 3.1.11). The long axis of the cells were in the same direction as of the collagen fibres (Fig. 3.1.12).

In B-APN treated rats, the alveolar crest fibres (Fig. 3.1.6) lacked the arrangement of well defined bundles seen in the controls. The collagen fibres here were aggregated in hyalinized areas of extracellular matrix of the periodontal ligament. The attachment of collagen fibres to the cemental surface appeared quite regular but the arrangement of collagen fibre insertion to the alveolar crest, was irregular in this material (Figs. 3.1.16, 17). The cells in this region appeared to have an arrangement with collagen fibres which was not quite similar to that observed in the controls.

The shape of the alveolar crest, site of the insertion of alveolar crest fibres, was conical in shape in the normal rats (Fig. 3.1.7). The crest of the alveolar bone, in the treated rats, was not of conical shape but was rather obtuse (Fig. 3.1.16).

In the region of horizontal and oblique fibres the collagen fibres, in the controls, were seen to run in well defined bundles between alveolar bone and cementum. These fibres were seen inserting into bone as well as cementum in sharp and well defined bundles (Fig. 3.1.8). The fibroblasts of this region were arranged randomly among the bundles of collagen fibres with their long axes in the direction of collagen fibres as described for other regions (Fig. 3.1.10). They were fusiform in shape

and presented a typical fibroblast-like appearance (Figs. 3.1.13, 14).

In the treated rats the region of horizontal and oblique fibres presented a picture (Fig. 3.1.15) which was drastically different as compared to the controls. The well defined bundles of collagen fibres running from alveolar bone to cementum, as seen in the controls, were absent (Figs. 3.1.18, 19). The fibroblasts in this region were not arranged in a random fashion but were aggregated into groups (Figs. 3.1.19, 20). These cells lay close to each other presenting a palisade arrangement with their long axes in the general direction of collagen fibres (Figs. 3.1.19, 20). These groups of cells were separated by the region of "hyalinized" extracellular matrix (Figs. 3.1.19, 20). These areas of "hyalinized" matrix, under high power, were found to be collagen fibres arranged in a random fashion without any definite orientation toward the surfaces of cementum or alveolar bone (Figs. 3.1.19, 20). The insertion of the collagen fibres in alveolar bone was seen to be only in certain areas opposite the areas of hyalinized matrix and not in a uniform pattern as in the controls (Fig. 3.1.21). The insertion of the fibres into cementum was, however, found to be comparable to the controls (Fig. 3.1.22).

The cells which formed the groups, when viewed under high power, were plump and oval as compared to the fusiform shaped cells in the control periodontal ligament (Fig. 3.1.23). These

cells showed intracellular vacuoles (Fig. 3.1.23).

The alveolar bone surface, in the controls, at the region of horizontal and oblique fibres was quite regular and did not show any cavitation of the interdental septae (Fig. 3.1.7).

In the B-APN treated rats the interdental septae of the alveolar bone did not present as smooth surfaces as seen in the controls (Fig. 3.1.15).

In the region of the root apex, the collagen fibres which extended from the tip of the root to the bottom of the socket of the alveolar bone showed a regular pattern of arrangement in the controls (Fig. 3.1.8). The fibroblasts also exhibited a pattern of arrangement in relation to collagen fibres similar to the one described for other regions of the periodontal ligament.

In the B-APN treated rats, the apical region showed the structures similar to the ones described in the region of horizontal and oblique fibres (Fig. 3.1.24). The cells were arranged in groups, although the groups were not as discrete as in the region of horizontal and oblique fibres, and were separated by areas of hyalinized matrix (Fig. 3.1.25). However, the effects were not as pronounced as in the region of the horizontal and oblique fibres.

3.1.3 ELECTRONMICROSCOPY

For ultrastructural analysis of the B-APN induced changes in periodontal ligament the regions of horizontal and oblique fibres and the region of apical fibres, the most effected areas seen under light microscope, were examined after preparing the tissue as described in materials and methods.

(i) Effect of B-APN on Cells

The region of horizontal and oblique fibres, in B-APN treated rats showed the fibroblasts lying in close approximation to each other forming groups. No special junctional complexes between these cells were discernible (Figs. 3.1.28, 29). The most striking changes in the ultrastructure of these cells were observed in their mitochondria. The latter were enlarged and appeared swollen. Quite often only a few cristae were discernible and sometimes no cristae were visible at all. The electron density of the mitochondrial matrix appeared to have decreased. The outer mitochondrial membrane always appeared complete and regular while the inner membrane always showed distortion.

The region of horizontal and oblique fibres, in controls, showed fibroblasts which were elongated and presented a normal fibroblast like appearance (Figs. 3.1.26, 27). They had a normal complement of organelles and showed no distortion of their mitochondria.

The region of the apical fibres of PDL of B-APN treated rats showed fibroblasts which appeared swollen and did not have a normal fusiform shape (Fig. 3.1.30). The mitochondria appeared enlarged and their cristae were frequently distorted (Fig. 3.1.30). These effects, however, were of lesser magnitude than those in the region of horizontal and oblique fibres.

The osteoblasts present in the PDL at the surface of alveolar bone, in B-APN treated rats, showed a normal cellular morphology. Their mitochondria appeared quite normal (Fig. 3.1.32). The cells which were away from the bone surface, well inside the periodontal ligament, showed more severe effects on their mitochondrial morphology (Fig. 3.1.31).

In order to assess the effects of fixation and decalcification, some muscle tissue was left attached to the jaw bones throughout the preparation procedure. These skeletal muscles did not show any effect on the morphology of their mitochondria (Fig. 3.1.33).

The fibroblasts of the various regions of the periodontal ligament in the controls as well as B-APN treated rats showed well developed rough surface endoplasmic reticulum. A slightly electron dense amorphous material was seen in the cisternal lumen. Sometimes, in the B-APN treated material, the cisternae were dilated but no consistent differences were discerned. The Golgi complex was quite extensive but again no consistent differences were apparent due to B-APN treatment (Figs. 3.1.26,

29, 30).

The cells, in controls as well as in the B-APN treated material, showed dense lysosome like bodies (Figs. 3.1.27, 29, 30). Those structures contained electron dense granular material. Though it is difficult to quantitate the number of these bodies based on an ultrastructural study, the number appeared to have increased in the B-APN treated material (Fig. 3.1.29).

In B-APN treated rats the areas of hyalinized matrix, as described in light microscopic observation, showed the collagen fibrils arranged in a manner without any orientation. Some fibrils were even seen to form loops (Fig. 3.1.34).

(ii) Effect of B-APN on Collagen Fibril Diameter

To quantitate the changes in the diameter of collagen fibrils, the electronmicrographs from the regions of "hyalinized matrix" from at least three animals were enlarged (final magnification X 1, 10,000) and the diameter of 100 collagen fibrils were measured. Similarly in the controls from the region of horizontal and oblique fibres, the diameter of 100 collagen fibrils were measured.

Frequency distribution of the diameter of collagen fibrils, in the controls as well as B-APN treated rats, is shown in figure 3.1.35. In the controls the most frequent diameter was 39 nm where as in the lathyrctic periodontal ligament it was 29.2 nm.

ACID PHOSPHATASE ACTIVITY (BURSTONE, 1958)

REGION	CONTROL	B-APN
<u>A. BONE</u> CREST OF I D SEPTA HORIZONTAL AND OBLIQUE FIBRES. APICAL FIBRES	- + +	+++ +++ ++
<u>B. PERIODONTAL LIGAMENT</u> ALVEOLAR CREST HORIZONTAL AND OBLIQUE FIBRES APICAL FIBRES	- - -	+++ +++ +++

3.1.4 HISTOCHEMISTRY

In order to evaluate the acid phosphatase activity semiquantitatively, in different regions of the periodontal ligament and the alveolar bone, 8 um thick paraffin sections were incubated for acid phosphatase reaction according to a method described by Burstone (1958). An incubation time of one hour was found to give most satisfactory results on these sections.

Although it is quite difficult to quantitate the enzymatic activities by visual microscopic examinations alone yet because of the marked differences between the two groups an attempt has been made to estimate the enzymatic activity on a scale of 0-4. These data are shown in the Table 3.1.1. A detailed description of the location of acid phosphatase reaction in various regions of the periodontal ligament and alveolar bone follows.

(i) General Distribution of Acid Phosphatase

Fig. 3.1.36 is a section through the mandible of the control rat. No appreciable amount of the reaction product of acid phosphatase is seen on the surface of alveolar bone and dentin. Fig. 3.1.37 is a low power micrograph of a section through the mandible of B-APN treated rat. The acid phosphatase activity is seen as a dark reaction product on the surface of alveolar bone, dentin and in addition in certain regions of the periodontal ligament.

(ii) Distribution in Different Regions

Region of alveolar crest fibres

There was no appreciable reaction product of this enzyme in the periodontal ligament as well as in the alveolar bone of the controls (Fig. 3.1.38).

In the B-APN treated rats, both the periodontal ligament and alveolar bone both showed the reaction for acid phosphatase activity. In the periodontal ligament the reaction product was diffused although certain specific spots were quite dark (Fig. 3.1.39). However, at the surface of the alveolar bone, the acid phosphatase activity was so intense that it was difficult to recognize the morphology of the stained cells (Fig. 3.1.39). These cells were relatively large and from their general appearance seemed to be osteoclasts. However, other cells which appeared to be osteoblasts were also positive for acid phosphatase. The osteocytes in their lacunae, also showed a positive but slight reaction for acid phosphatase (Fig. 3.1.40).

Region of horizontal and oblique fibres

The periodontal ligament and alveolar bone of the controls, in the region of horizontal and oblique fibres, showed reaction product for acid phosphatase which was present at certain spots on dentin and alveolar bone surfaces. Some cells in the periodontal ligament were however positive. At the alveolar bone surface, the large cells, osteoclasts, showed intense reaction product and were occupying small cavities, the

Howship's lacunae (Fig. 3.1.40). The osteocytes on the other hand did not show any appreciable amount of acid phosphatase reaction product (Fig. 3.1.40).

In the B-APN treated rats, the region of horizontal and oblique fibres, showed intense reaction product both on the surfaces of alveolar bone as well as in the periodontal ligament. In the latter this activity was mainly in the cells, fibroblasts, which show a palisade arrangement and formed groups (as described in light microscopic observation), Fig. 3.1.41. Also some other cells present in the periodontal ligament, which were not arranged in groups, showed dark reaction product (Fig. 3.1.41).

On the surface of the alveolar bone, the large cells which appeared to be osteoclasts, showed intense reaction for acid phosphatase (Fig. 3.1.41). The osteoblasts on the bone surface also showed the activity of acid phosphatase but the reaction product was not as intense as in the osteoclasts (Fig. 3.1.41). The osteocytes were also positive for acid phosphatase reaction (Fig. 3.1.42).

Region of apical fibres

The controls, in the region of apical fibres, showed no reaction product in the periodontal ligament whatsoever (Fig. 3.1.42).

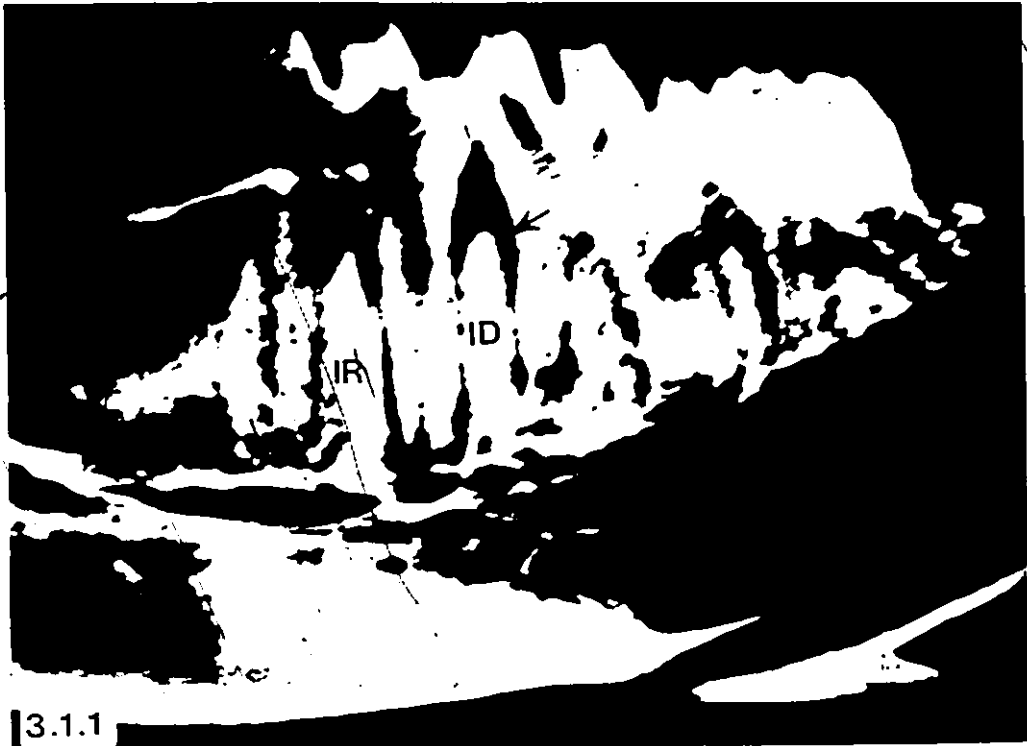
The only reaction product seen in this region was on the alveolar bone surface in large cells, the osteoclasts (Fig.

3.1.42).

In B-APN treated rats, the acid phosphatase activity was again present both in the periodontal ligament as well as on the alveolar bone surfaces (Fig. 3.1.43). In the periodontal ligament, the fibroblasts showed a positive reaction for acid phosphatase however the activity was not as intense as seen in the fibroblasts of the region described above (Fig. 3.1.43).

On the alveolar bone surfaces, large cells, presumably osteoclasts, stained very darkly for acid phosphatase activity. The osteoblasts and osteocytes also showed dark reaction product for acid phosphatase (Fig. 3.1.43).

The rat kidney sections, kept as control for the histochemical reaction, showed the reaction product of acid phosphatase to be present in the cells of the proximal as well as the distal convoluted tubules (Fig. 3.4.12).



3.1.1



3.1.2

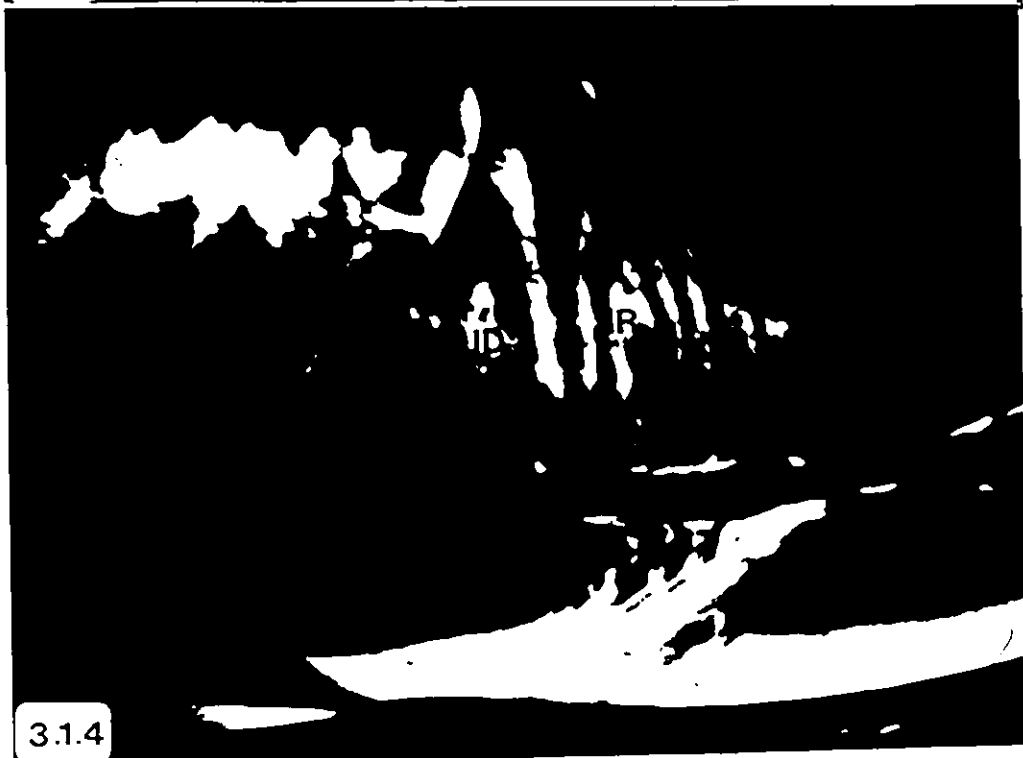
Both figures from the controls.

Fig. 3.1.1 A microradiograph of the control rat hemimandible taken from buccal view. Area of molar teeth is seen in the microradiograph. Between the two roots of first molar interradicular septa is marked as IR. In between first and second molars interdental septa of alveolar bone is marked as ID. Note the radioopacity of interradicular and interdental septae. Arrow point toward the conical shape of Interdental septae. Also note the height of Interdental septa X 20.

Fig. 3.1.2. A microradiograph similar to Fig. 3.1.1. but taken from the lingual view. ID denotes interdental septa while IR is interradicular septa. Arrow again points toward conical shape of interdental septa X 20.



3.1.3



3.1.4

Both figures from the B-APN-treated group.

Fig. 3.1.3. A microradiograph from hemimandible of B-APN-fed rat showing the area similar to Figs. 3.1.1. and 3.1.2. Interradicular septa between the roots of first molar is marked IR while Interdental septa is marked ID. Arrow points towards a blunt tip of Interdental septa. Note the reduced radiodensity as compared to the figures 3.1.1. and 3.1.2. There appears to be a general reduction in the bone tissue X 20.

Fig. 3.1.4. A microradiograph similar to Fig. 3.1.3. but taken from lingual view. Interradicular (IR) and Interdental (ID) septae are seen here. Note the radiolucency and blunt tip of Interdental septa X 20.



3.1.5



3.1.6




Fig. 3.1.5. A microradiograph of a part of longitudinal section through the control rat hemimandible. Note the radiodensity and general presence of bone tissue X 50.





Fig. 3.1.6. A microradiograph similar to Fig. 3.1.5. but from the rat fed with B-APN. Note the increased radiolucency of bone, increased size of marrow and nutritive spaces and a general reduction in amount of bony tissue X 50.





31.7



31.8



31.9



31.10

All from the controls.

Fig. 3.1.7. Photomicrograph of part of the longitudinal section through periodontal ligament and alveolar bone of the normal rat showing dentin (D), alveolar bone (b). Arrow points toward alveolar crest and arrow head towards transseptal fibres X 130.

Fig. 3.1.8 A section similar to Fig. 3.1.7. The microphotograph shows dentin (D), Cementum (C), Pulp (PU), and the region of apical fibres (AP), alveolar bone (b), and region of oblique fibres. X 130.

Fig. 3.1.9. A higher power photomicrograph showing the insertion of alveolar crest fibres into alveolar crest (arrow). Photomicrograph also shows dentin (D) and alveolar bone (b) X 326.

Fig. 3.1.10. A higher power photomicrograph of region of oblique fibres (Ob. F.) in periodontal ligament of the control rats. Note the uniform pattern of cells and collagen fibre arrangement X 326.



Fig. 3.1.11. A high power photomicrograph of alveolar crest fibre region of the control rat showing insertion of the alveolar crest fibres (ALV) into bone (b). Dentin (D) is towards the lower portion of micrograph X 522.

Fig. 3.1.12. A high power photomicrograph of the control rat periodontal ligament in the region of alveolar crest. Insertion of collagen fibrils towards cementum of root is seen and dentin (D) toward the left side of photomicrograph while the bone (not seen here) is towards right side. Note the regular arrangement of cells and collagen fibres X 822.

Fig. 3.1.13. Region of oblique fibres (Obl.F.) in the control rat periodontal ligament. Insertion of collagen fibrils in this region is seen (arrow heads) into the the alveolar bone (b) X 522.

Fig. 3.1.14. A higher power photomicrograph of the region seen in Fig. 3.1.13. The bundles of collagen fibrils (arrow heads) are seen inserting into alveolar bone (b). The dentin (not seen) is towards the left side of photomicrograph X 822.



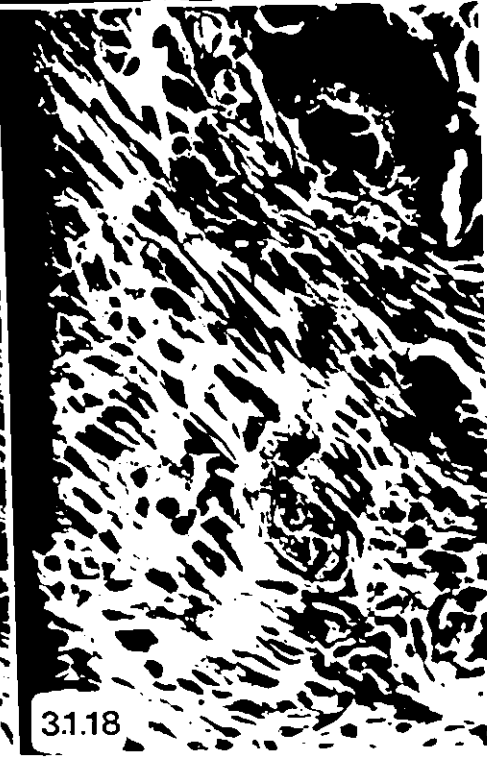
3.1.15



3.1.16



3.1.17



3.1.18

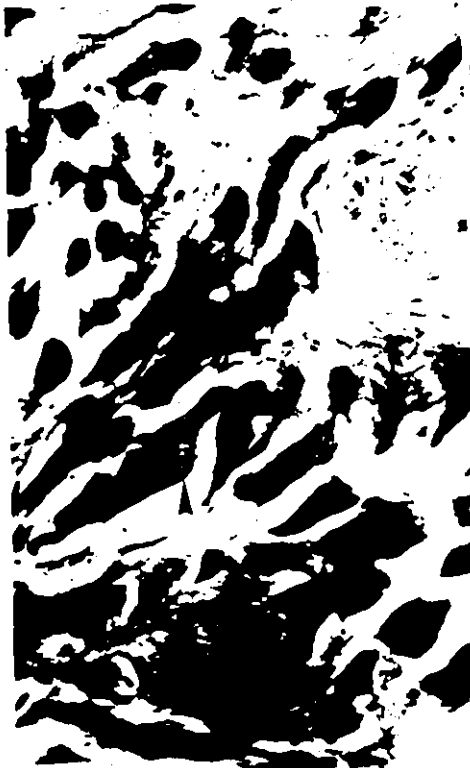
All photomicrographs are from the B-APN treated group.

Fig. 3.1.15. A part of the longitudinal section through the mandible of rat treated with B-APN. Region of transeptal fibres (tf), alveolar crest fibres (alv.), oblique fibres (Obl) and apical fibres region (ap) is seen. Small arrow heads point towards the hyalinized areas of extracellular matrix of the periodontal ligament X 130.

Fig. 3.1.16. Region of transeptal fibres (TF) and alveolar crest fibres (ALF) are seen. Note the normal arrangement of collagen fibres and cells in transseptal fibre region but hyalinization of extracellular matrix in region of oblique fibres (arrow head). Dentin (D) is at the right side while alveolar bone (b) is in the lower left corner X 326.

Fig. 3.1.17. A high power photomicrograph from the region of transseptal fibres, Dentin (D) is in upper right corner. While alveolar bone (b) is in lower left corner. Note the regular arrangement of collagen fibres and cells in this region X 522.

Fig. 3.1.18. Region of oblique and horizontal fibres from the B-APN treated rat periodontal ligament. Groups of cells (arrows), hyalinization of extracellular matrix (arrow heads), alveolar bone (b) and Dentin (D) are seen in this region X 522.



3.1.19



3.1.20



3.1.21



3.1.22

All photomicrographs are from the B-APN treated group.

Fig. 3.1.19. A high power photomicrograph from the region of horizontal and oblique fibres of B-APN fed rats. The cells in groups (arrow heads) and hyalinization of extracellular matrix (arrow) are seen X 522.

Fig. 3.1.20. A photomicrograph similar to Fig. 3.1.19. Cellular groups (arrow heads) and hyalinized areas of extracellular matrix (arrows) are seen. Note the irregular pattern of collagen fibres in the hyalinized areas. The regular pattern of the control material (seen in Figs. 3.1.13 and 3.1.14) is lacking in this material X 522.

Fig. 3.1.21. Region of oblique fibres of the B-APN fed rat periodontal ligament. Insertion of collagen fibrils (small arrow) into alveolar bone (b) are seen. Cellular groups (arrow head) and the hyalinized area of extracellular matrix (big arrow) are also seen. Note that insertion pattern is quite different from that seen in the control material (as seen in Fig. 3.1.13) X 522.

Fig. 3.1.22. Region of oblique fibres. Insertion of the PDL collagen fibres into cementum is seen (small arrow). Dentin (D), cellular groups (arrow heads) and the hyalinized areas of extracellular matrix are also seen X 522.

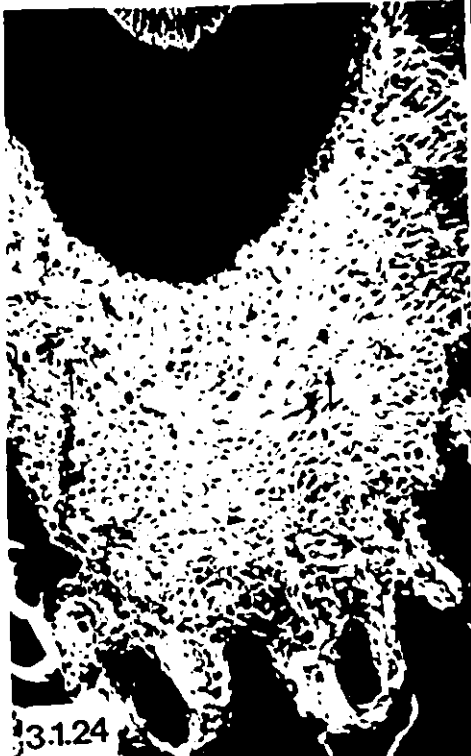


Fig. 3.1.23 A photomicrograph of the region of oblique fibres of the B-APN fed rats. The groups of cells (big arrows), hyalinized areas of extracellular matrix (big arrow heads) and intracellular vacuoles (small arrow heads) are seen X 600.

Fig. 3.1.24. Region of the apical fibres of B-APN fed rat periodontal ligament. Root of a tooth (RT), alveolar bone (b) and hyalinized areas of extracellular matrix (arrows) are seen X 180.

Fig. 3.1.25. A higher power photomicrograph of the area seen in Fig. 3.1.24. Areas of the hyalinized matrix (arrows) are also seen. Note that extent of the alteration in pattern of arrangement of cells and collagen fibres, is not as severe as seen in the region of oblique fibres (Figs. 3.1.19 and 3.1.20) X 326.



3.1.26

Fig. 3.1.26. A section through the region of oblique fibres of the normal rat periodontal ligament. Several fibroblasts and collagen fibril (CF) in between the cells are seen. In fibroblasts, rough surface endoplasmic reticulum (er), mitochondria (arrow heads), and Golgi complexes (arrows) are seen. Note the random arrangement of fibroblasts and collagen fibrils. Mitochondria (arrow heads) show the normal morphology X 16,100.

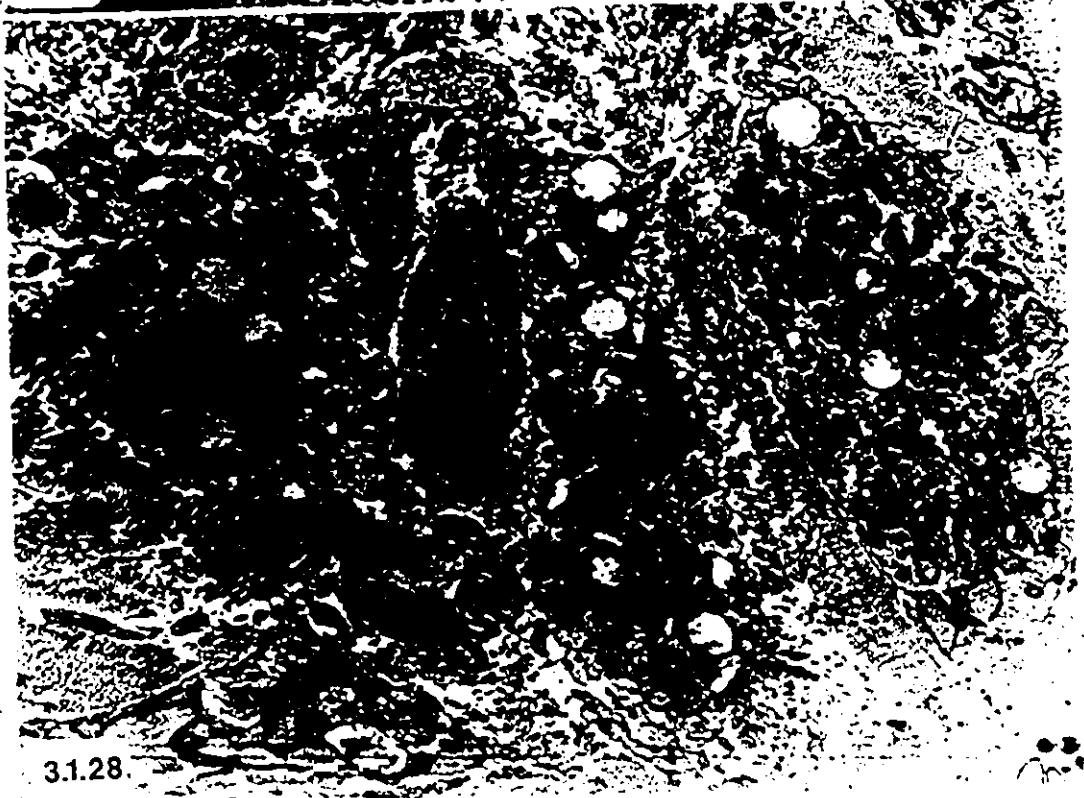
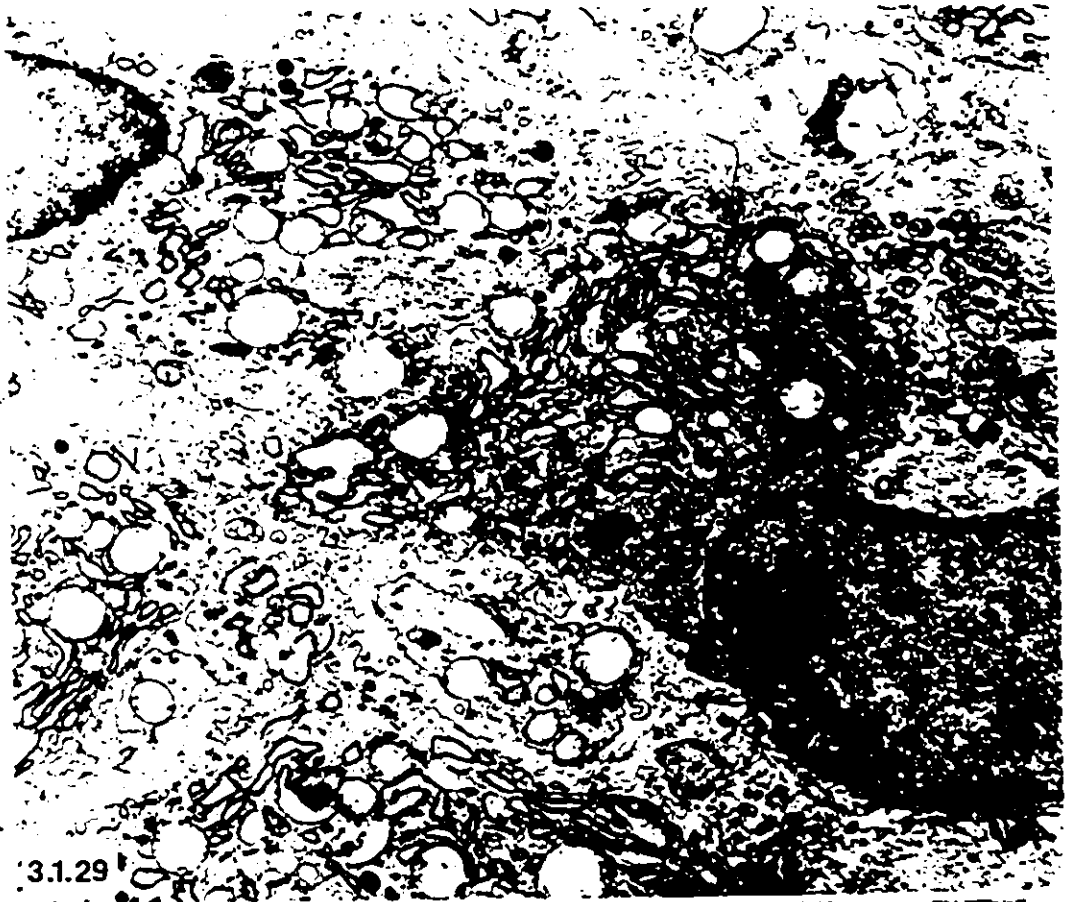
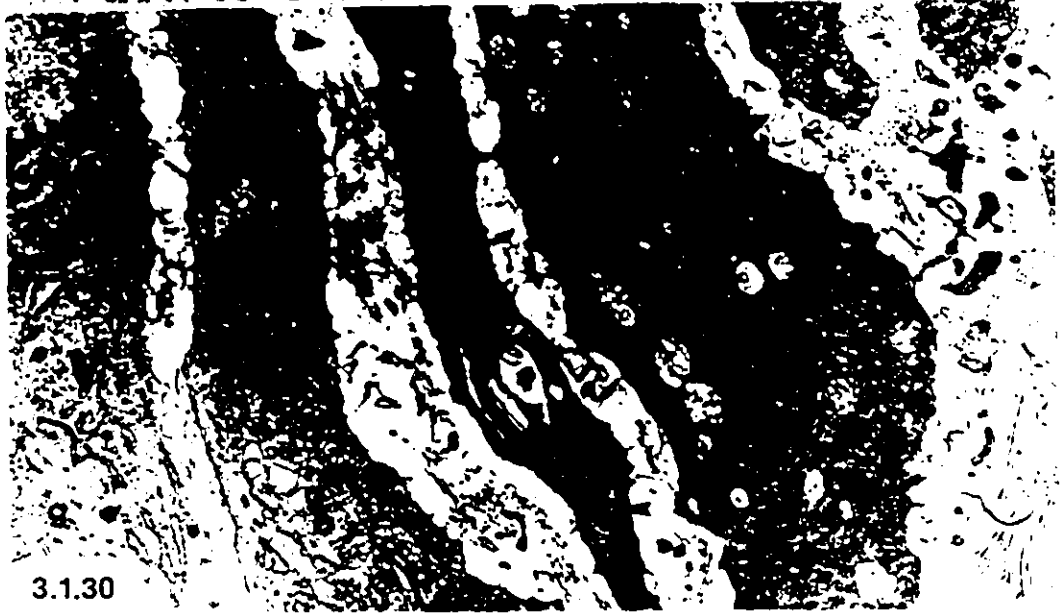


Fig. 3.1.27. A lower power electronmicrograph of PDL from the normal rats... Several fibroblasts and collagen fibrils (CF) in between the cells are seen. Mitochondria (arrow heads) show a normal morphology X 6,394.

Fig. 3.1.28. An electronmicrograph of a section through the region of oblique fibres of the B-APN fed rats. Parts of four fibroblasts are seen. In between the cells are collagen fibrils (CF). The fibroblasts show mitochondria (arrow heads) with vacuolization of their matrix and dilated cisternae of the rough surface endoplasmic reticulum (er) X 7,980.



3.1.29



3.1.30

Both electronmicrographs from the B-APN treated group.

Fig. 3.1.29. A high power electronmicrograph of the area similar to one seen in Fig. 3.1.28. In fibroblasts, note the distorted mitochondrial morphology (arrow heads), dilated rough surface endoplasmic reticulum (er) but a normal Golgi complex (g). In extracellular matrix several bundles of oxytalin fibrils (Of) are also seen X 11,400.

Fig. 3.1.30. A section through the region of apical fibres of the B-APN fed rat periodontal ligament. Several fibroblasts are seen. In the fibroblasts mitochondria (big and small arrow heads), rough surface endoplasmic reticulum (er) and Golgi complexes (g) are seen. The extent of distortion of mitochondrial morphology is variable in different cells. The mitochondria more severely affected are marked by small arrow heads while those which have less affects are marked by the large arrow heads. Note that in this region the effect is not as severe as seen in the region of oblique fibres (Fig. 3.1.29) X 11,400.



bone

3.1.31



3.1.32



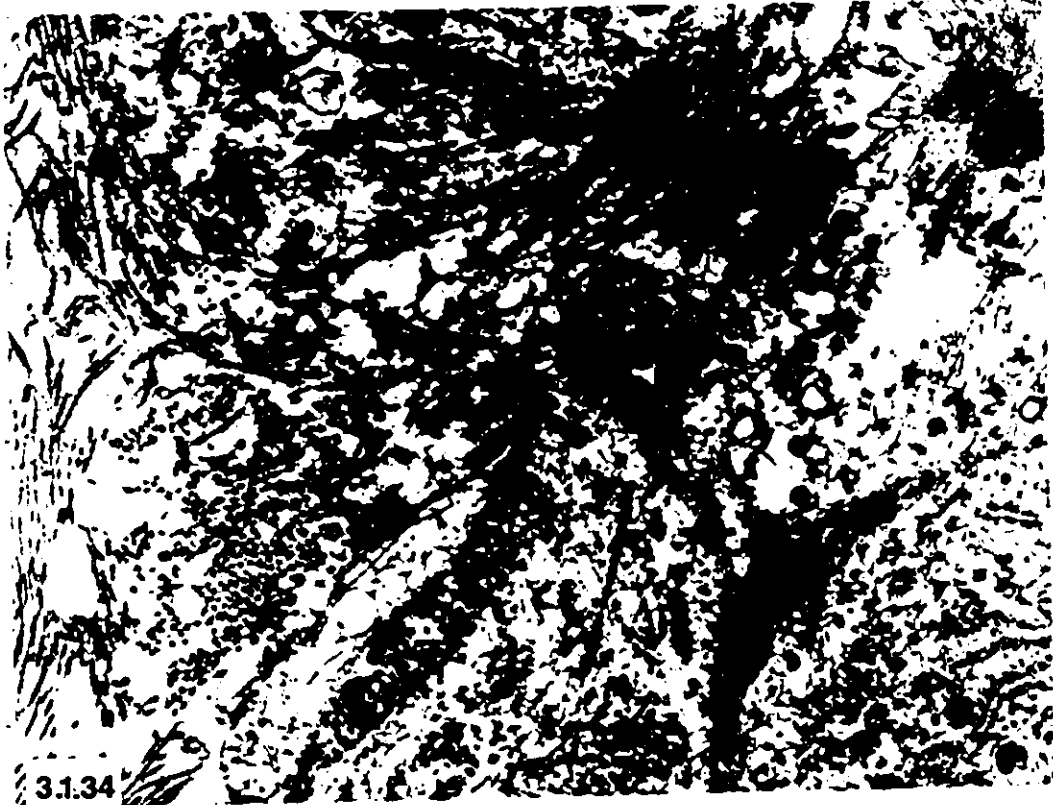
3.1.33

All electronmicrographs from the B-APN treated group.

Fig. 3.1.31. Region of oblique fibres of B-APN fed rats. The fibroblasts well inside the periodontal ligament (PDL) show distorted mitochondria (arrows) while a cell towards the alveolar bone has no effect on the mitochondrial morphology (arrow heads). Alveolar bone is also seen (Bone) X 11,400.

Fig. 3.1.32. An osteoblasts with normal mitochondrial morphology (arrow heads) is seen at the alveolar bone (bone) surface. Collagen fibrils with their characteristic banding pattern are also seen X 10,260.

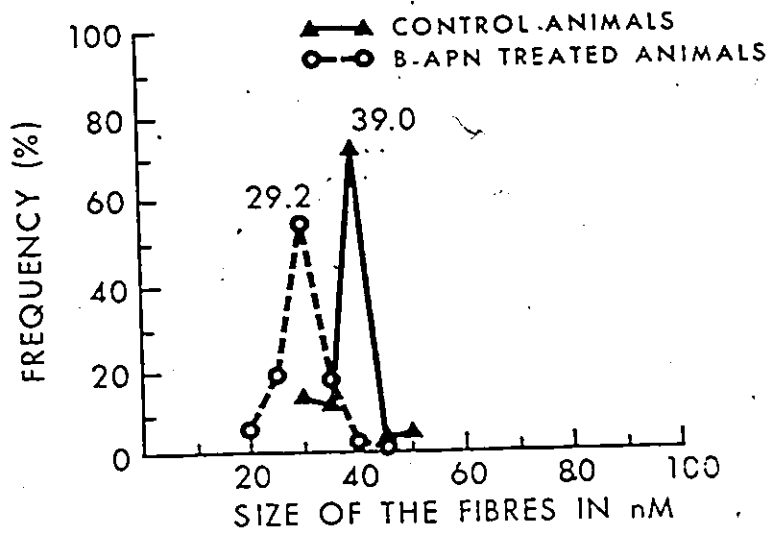
Fig. 3.1.33. Skeletal muscle cells, prepared and processed like periodontal ligament showing normal morphology of mitochondria (arrow heads). X 51,300.



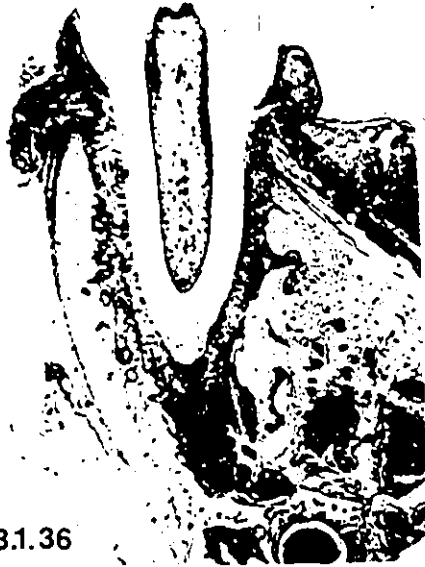
3.1.34

Fig. 3.1.34. Area of the hyalinized extracellular matrix in region of oblique fibres from the periodontal ligament of B-APN fed rats. Collagen fibrils (CF) and oxytalin fibrils (Of) are seen. The disoriented and looped collagen fibrils (arrows) are also seen X 17,100.

FREQUENCY DISTRIBUTION OF FIBRES IN PERIODONTAL LIGAMENT OF RAT



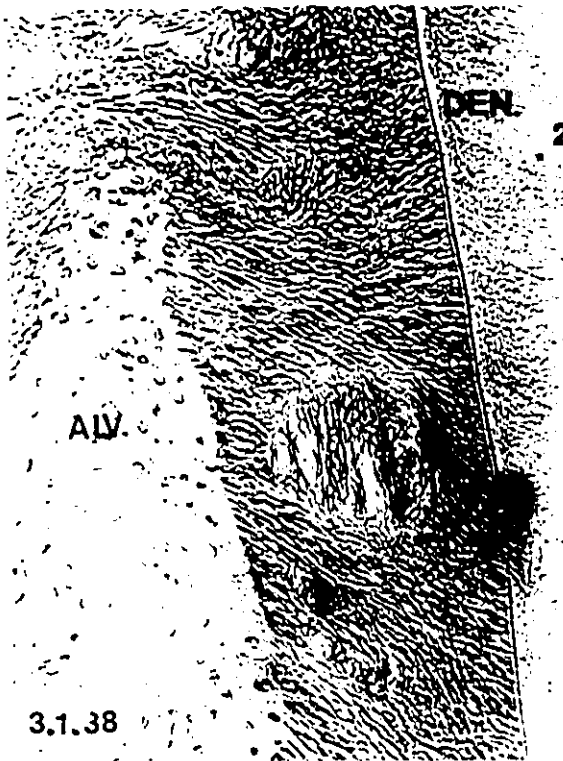
3.1.35



3.1.36



3.1.37



3.1.38



3.1.39

Fig. 3.1.36.-A low power photomicrograph of section of normal rat jaw. Alveolar bone, periodontal ligament, and a tooth is seen. The sections have been incubated for acid phosphatase localization (Burstone's method) X 75.

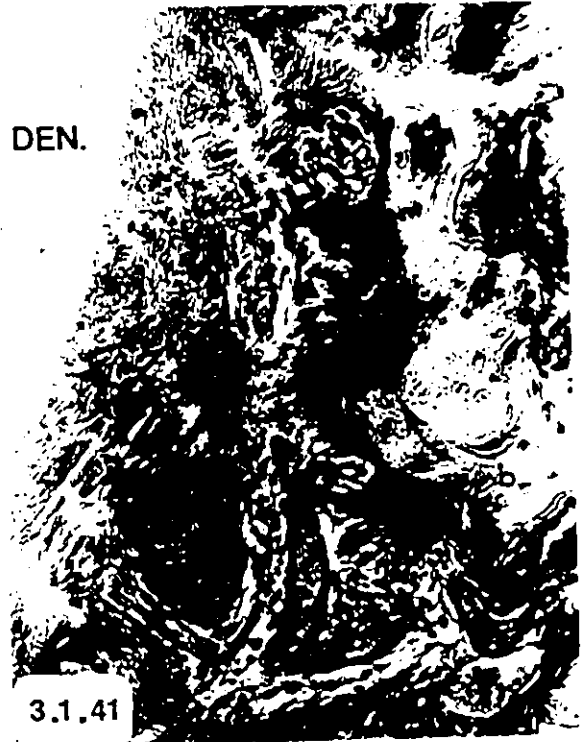
Fig. 3.1.37. A section similar to Fig. 3.1.36 but from the jaw of a rat fed with B-APN, incubated for acid phosphatase localization. Note the dark reaction product at the surface of alveolar bone X 75.

Fig. 3.1.38. Region of alveolar crest fibres of the normal rat periodontal ligament. Alveolar bone (ALV) does not show any appreciable reaction product. Periodontal ligament is also negative. Reaction product is seen only in the large cells (arrow) at discrete spots X 588.

Fig. 3.1.39. A photomicrograph of an area similar to Fig. 3.1.39 but from a B-APN fed rat. Note the dark reactive product (arrow) on the surface of alveolar bone (ALV) X 588.



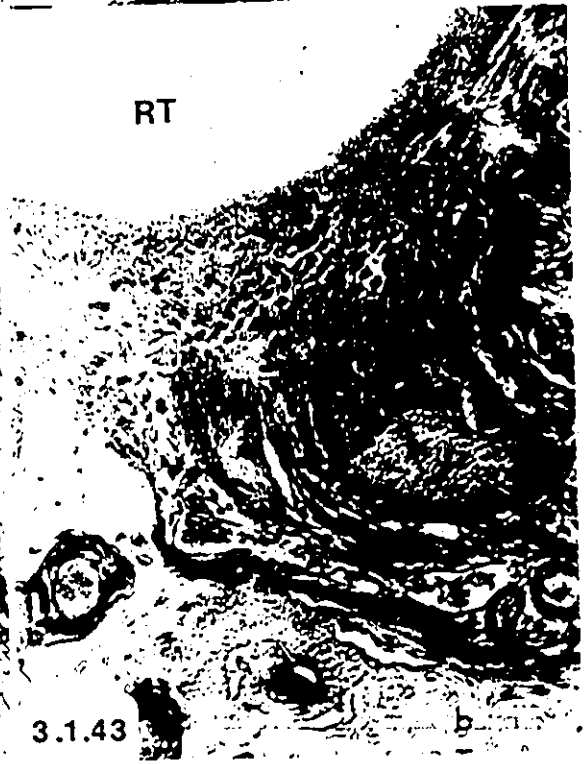
DEN.



DEN.



RT



RT

Fig. 3.1.40. Region of oblique fibres from the periodontal ligament of a normal rat. The section has been incubated for acid phosphatase reaction (Burstone's method). The dark reaction product is present at the discrete spots on alveolar bone (b) surface and in the periodontal ligament. The Dentin of root is marked by letters (DEN) X 588.

Fig. 3.1.41. A section similar to Fig. 3.1.41 and incubated in a similar manner but from the B-APN fed rat. Note the dark reaction product (arrow) at the surface of alveolar bone (b) and in the cells forming groups in periodontal ligament (hollow arrow). Also note that the osteocytes show dark reaction product X 588.

Fig. 3.1.42. Region of apical fibres from the periodontal ligament of a normal rat. The section has been incubated for acid phosphatase (Burstone's method). Root of the tooth (RT) and alveolar bone (b) are seen. The dark reaction product is only present in the large cells (presumably osteoclasts) at the surface of bone (arrow) X 588.

Fig. 3.1.43. A section similar to Fig. 3.1.43 but from a B-APN fed rat. The dark reaction product is seen on the alveolar bone (b) surface (arrow) and in cells of periodontal ligament (hollow arrow) X 588.

3.2 EFFECTS OF B-AMINOPROPIONITRILE ON BONE MATRIX FORMATION AND ITS MINERALIZATION IN RATS

The studies were done on rats as well as chicks. The investigation on chicks is discussed separately in section 3.3. This investigation involved rats. These were four week old males of Sprague Dawley Strain. Their mean body weight was recorded at the start of the experiment and was 69 ± 2 gms. They were divided into two groups. One group was fed a normal diet (Purina Rat Chow, Ralston Purina Co., St. Louis, Missouri, U.S.A.), the second group (experimental) was fed Purina Chow plus B-APN (0.25%).

3.2.1 Effects on General Body Growth and Tibial Length

To see the effect of B-APN on the general body growth and on bones at organ level the body weight and tibial lengths of the controls and B-APN rats were recorded at 5, 10 and 20 day intervals during the experiment. The body weight of the controls was found to be 81.80 ± 0.374 gm, 102.80 ± 1.113 gms and 161.40 ± 7.406 gms at 5, 10 and 20 days respectively. The body weight of the B-APN treated rats was 81.00 ± 0.836 gm, 99.20 ± 1.356 gms and 153.80 ± 3.611 gms at 5, 10 and 20 days respectively.

The lengths of tibias of the control rats were 2.76 ± 0.024 cm, 2.96 ± 0.023 cm and 3.214 ± 0.01094 cm, while those of the rats treated with B-APN were 2.75 ± 0.032 cm, 2.94 ± 0.040 cm and 3.220 ± 0.0217 cm at 5, 10 and 20

TABLE 3.2.1.

BODY WEIGHT AND TIBIAL LENGTH OF CONTROLS AND B-APN TREATED RATS^a

TIME *	PARAMETER	CONTROL ANIMALS	B-APN TREATED ANIMALS	P ^b
5 days plus 36 hours	1. Body Weight	81.80 ± 0.374 ^c gms	81.00 ± 0.836 gms	NS ^d
	2. Tibial Length	2.76 ± 0.024 cm	2.75 ± 0.032 cm	NS
10 days plus 36 hours	1. Body Weight	102.80 ± 1.113 gms	99.20 ± 1.356 gms	NS
	2. Tibial Length	2.96 ± 0.023 cm	2.94 ± 0.040 cm	NS
20 days plus 36 hours	1. Body Weight	161.40 ± 7.406 gms	153.80 ± 3.66 gms	NS
	2. Tibial Length	3.214 ± 0.0194 cm	3.220 ± 0.0217 cm	NS

All data are based on observations from at least 5 rats.

a. Diet 0.25% B-APN

b. probability estimated by Students' T-Test

c. Mean ± S.E.

d. Not statistically significant at 0.05 probability level.

* Days denote the length of B-APN treatment and the hours denote the time after dosing with the labels. Thus total time of treatment equals days plus 36 hours.

days respectively.

These data is also shown in Table 3.2.1. The values represent the mean \pm standard error of the means for data from 5 rats.

The incorporation of ^3H -Proline, as a measure of bone matrix formation, and that of ^3H -Tetracycline, as a measure of mineralization, in the controls and B-APN treated rat bones was analyzed. The rationale for the use of these isotopes is discussed on page 178. This study was done in three phases.

3.2.2 ^3H -Proline and ^3H -Tetracycline Dynamics

In the first phase, to determine the time by which the ^3H -Proline and ^3H -Tetracycline become stabilized in bones, the normal rats were injected intraperitoneally either with ^3H -Tetracycline (3 uCi/100 gm body weight, sp. activity 570 uCi/mol), or with ^3H -Proline (10 uCi/60 gm body weight, sp. activity 248 uCi/n mol). The animals were sacrificed at 6, 12, 18, 24, 36 and 48 hours after dosing. The equal lengths of their tibial diaphyses were prepared for liquid scintillation counting as described in the materials and methods. The CPM (counts per minute)/100 mg. wet tibial weights were calculated.

The radioactivity left in normal rat bones at different time intervals after administration of ^3H -Tetracycline and ^3H -Proline is shown in Figures 3.2.1, 2. Initially the CPM/100 mg wet tibial weight were very high but as the time

elapsed the counts decreased and became stable at about 36 hours and did not change appreciably thereafter. On basis of this experiment the sacrifice time of the rats was decided to be 36 hours. (for further discussion of this point see page 178) after administration of isotopes, in all further investigations.

3.2.3 Effects of B-APN on Dynamics of ^3H -Proline and ^3H -Tetracycline

A similar experiment was conducted with the rats which had been fed a diet containing B-APN (0.25%) for 20 days to see if B-APN might have any effect on the time of stabilization of radio activities of the two isotopes. The radioactivity left in the bones of these rats, at different time intervals after administration of ^3H -Tetracycline and ^3H -Proline is shown in Figures 3.2.3 and 4. The results of these experiments were quite similar to the one conducted on normal rats.

3.2.4 Effect of Dose Variation of ^3H -Tetracycline on Radioactivity in Bones

Tetracycline has a very high affinity for bone because of its capability of making complexes with calcium ions at the bone surface. In order to evaluate the effects of different doses of tetracycline on the amount of radioactivity obtained 36 hours after administration, the normal rats were injected with two doses of ^3H -Tetracycline varying by a factor of ten (1.7 and 17 $\mu\text{Ci}/100$ gm body weight). The CPM/100 mg wet tibial weight, obtained at 36 hours, with two doses are shown in Fig. 3.2.5.

TABLE 3.2.2.

³H-TETRACYCLINE INCORPORATION AND ³H-PROLINE INCORPORATION IN CONTROL AND B-APN TREATED RATS^a

TIME*	PARAMETERS	CONTROL ANIMALS	B-APN TREATED ANIMALS	p ^b
5 days plus 36 hours	1. ³ H-Tetracycline Incorporation	2.02 ± 0.187 % ^c	1.21 ± 0.018 %	<.02
	2. ³ H-Proline Incorporation	1.193 ± 0.055 %	1.096 ± 0.055 %	NS
10 days plus 36 hours	1. ³ H-Tetracycline Incorporation	0.833 ± 0.0148 %	0.574 ± 0.0128 %	<.001
	2. ³ H-Proline Incorporation	0.270 ± 0.002 %	0.261 ± 0.008 %	NS
20 days plus 36 hours	1. ³ H-Tetracycline Incorporation	0.474 ± 0.0108 %	0.406 ± 0.009 %	<.01
	2. ³ H-Proline Incorporation	0.349 ± 0.012 %	0.327 ± 0.012 %	NS

All data are based on observations from at least 3 rats.

a. Diet 0.25% B-APN

b. probability estimated by Students' T-Test

c. Mean + S.E.

d. Not statistically significant at 0.05 probability level.

* Days denote the length of B-APN treatment and the hours denote the time after dosing with the labels. Thus total time of treatment equals days plus 36 hours.

On the basis of this experiment it was decided that a small dose is sufficient to obtain high enough counts.

3.2.5 Effects of B-APN on ^3H -Proline and ^3H -Tetracycline Incorporation

In the final phase of this isotopic incorporation investigations, one group of animals was fed a diet containing B-APN (0.25%) whereas the controls were fed a diet described above. Three rats from each group were injected either ^3H -Tetracycline (3 uCi/100 gm body weight) or ^3H -Proline (10 uCi/100 gm body weight) at 5, 10 and 20 days after the start of the experiment.

The CPM/100 mg wet tibial weight was calculated from the equal lengths of tibial diaphyses of the rats sacrificed thirty-six hours after the administration of ^3H -Proline and ^3H -Tetracycline by preparing their bones for liquid scintillation as described earlier. The incorporation of ^3H -Proline and ^3H -Tetracycline was then calculated as a percentage of the initial dose administered and is shown in Table 3.2.2. The values represent the mean \pm standard error of means for bones from 3 rats.

At day 5, the incorporation of ^3H -Tetracycline into bones of B-APN fed rats was $1.21 \pm 0.018\%$ while that of controls was $2.02 \pm 0.187\%$. The incorporation of ^3H -Proline into bones of controls and B-APN fed rats was $1.193 \pm 0.055\%$ and $1.096 \pm 0.055\%$ respectively.

At day 10, the incorporation of ^3H -Tetracycline in the

controls and B-APN fed rats was $0.833 \pm 0.0148\%$ and $0.674 \pm 0.0128\%$ respectively while that of ^3H -Proline was $0.270 \pm 0.002\%$ and $0.261 \pm 0.008\%$.

At day 20 the percentage of ^3H -Proline and ^3H -Tetracycline incorporated into the bones of the controls and B-APN fed rats was $0.349 \pm 0.012\%$, $0.327 \pm 0.012\%$ and $0.474 \pm 0.0108\%$, $0.406 \pm 0.009\%$ respectively.

Figure 3.2.6 shows a comparison of the bone matrix synthesis as shown by ^3H -Proline incorporation, and the mineralization, indicated by ^3H -Tetracycline incorporation in the controls and B-APN treated rats.

3.2.6 Effects of B-APN on Linear Bone Apposition

To obtain a correlation of morphological technique with the radioisotopic technique, the linear bone apposition was measured with the help of tetracycline fluorescent labelling technique. The sections obtained from tibial diaphyses at tibiofibular synostosis as described in the materials and methods, from rats sacrificed 36 hours after tetracycline injections at 5, 10 and 20 days, were examined under UV light for fluorescent microscopic measurements. With fluorescent microscopy two parameters were measured. 1: the width of bone matrix present between each two consecutive labels (0-5; 5-10; 10-20) from cold tetracycline administered materials at 0, 5, 10 and 20 days. 2: The width of the bone between the labels at 0 day and that of day 20 (0-20).

TABLE 3.2.3.

WIDTH OF BONE FORMED BETWEEN EACH TIME INTERVAL AND TOTAL WIDTH OF BONE FORMED THROUGHOUT THE EXPERIMENT

TIME	LINEAR BONE APPOSITION BETWEEN INTERVALS	P ^b
0-5 days control B-APN treated	52.29 ± 1.500 ^a 51.41 ± 2.190	NS ^c
5-10 days control B-APN treated	46.58 ± 0.508 45.70 ± 0.716	NS
10-20 days control B-APN treated	67.68 ± 2.092 65.48 ± 1.665	NS
0-20 days control B-APN treated	173.59 ± 1.502 171.84 ± 1.106	NS

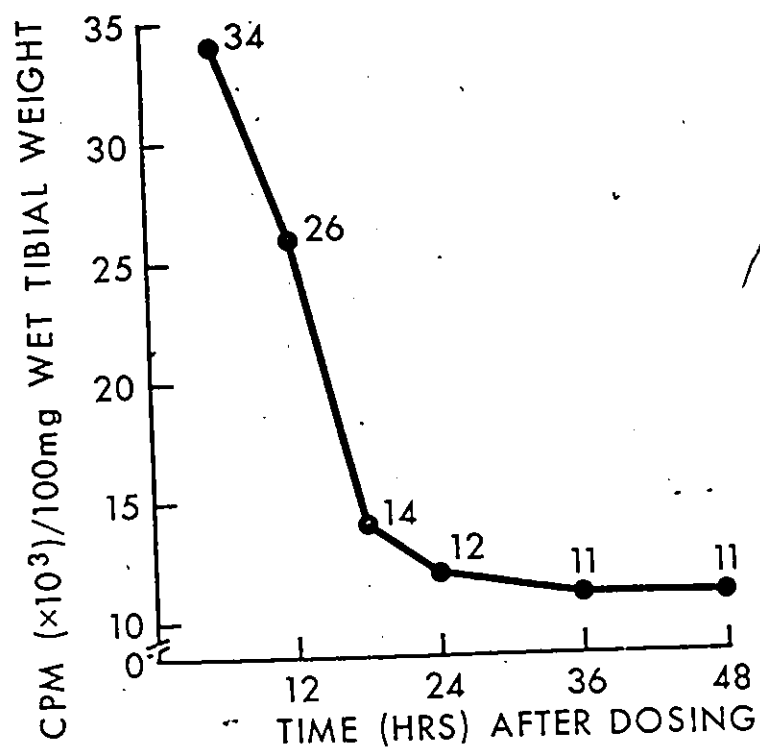
All measurements in um and taken at X 60

- a. Mean + S.F.
- b. Probability estimated by Students' T-Test
- c. Not significant at 0.05 probability level.

The linear apposition of bone between each time interval and the total linear bone apposition between the start of experiment and its termination is shown in Table 3.2.3. The values represent mean \pm standard error of the means for data from at least five cross sections from different rats.

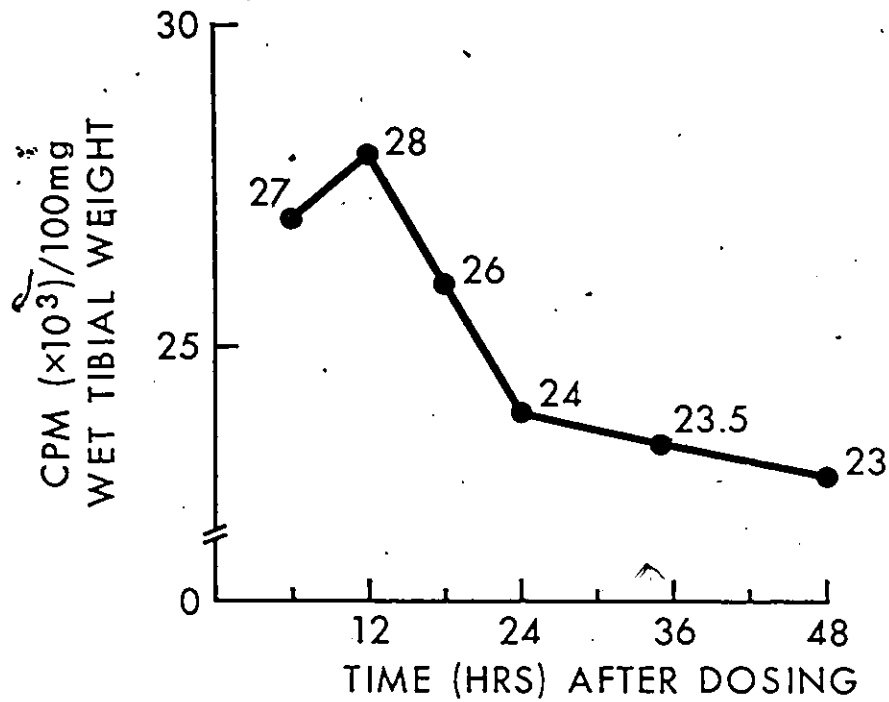
The Figures 3.2.7, 9 and 11 are from the control rats while Figures 3.2.8; 10 and 12 are from the rats treated with B-APN. The Figures 3.2.7 and 8 show the bands from cold tetracycline administration at 0 and 5 days. Figures 3.2.9 and 10 show 3 bands from 0, 5 and 10 day cold tetracycline administration. Figures 3.2.11 and 12 show four labels from cold tetracycline administered at 0, 5, 10 and 20 days. The first label from cold tetracycline given at the start of experiment (0 day) is seen on the endosteal surface. The distribution and intensity of the tetracycline labels were found to be similar in the controls and B-APN treated rats. The differences in intensity seen in various photomicrographs are not real as there is always some loss of fluorescence during UV study.

³H-TETRACYCLINE ACTIVITY REMAINING IN RAT TIBIA
AT DIFFERENT TIMES (HRS) AFTER DOSING



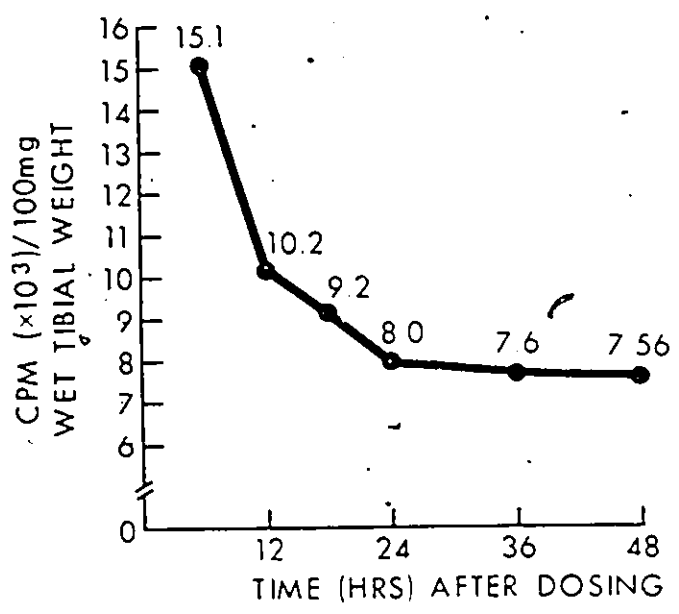
3.2.1

³H-PROLINE ACTIVITY REMAINING IN RAT TIBIA AT
DIFFERENT TIMES (HRS) AFTER DOSING



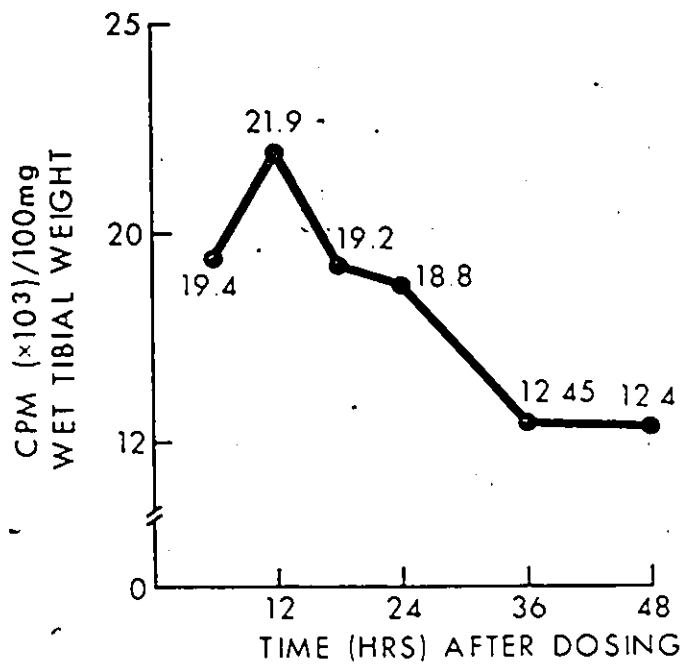
3.2.2

³H-TETRACYCLINE ACTIVITY REMAINING IN RAT
(TREATED WITH β-APN FOR 3 WEEKS) TIBIA
AT DIFFERENT TIMES (HRS) AFTER DOSING

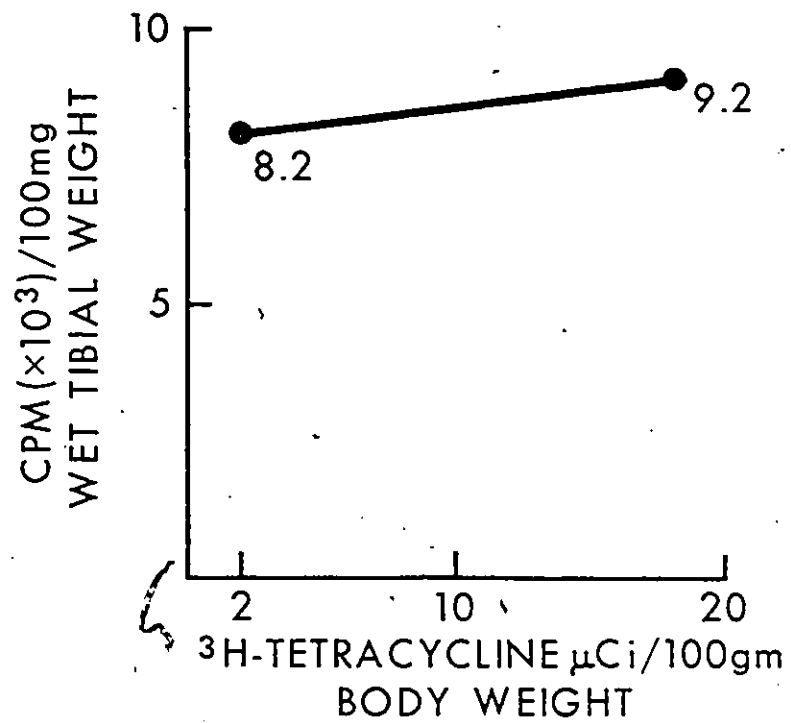


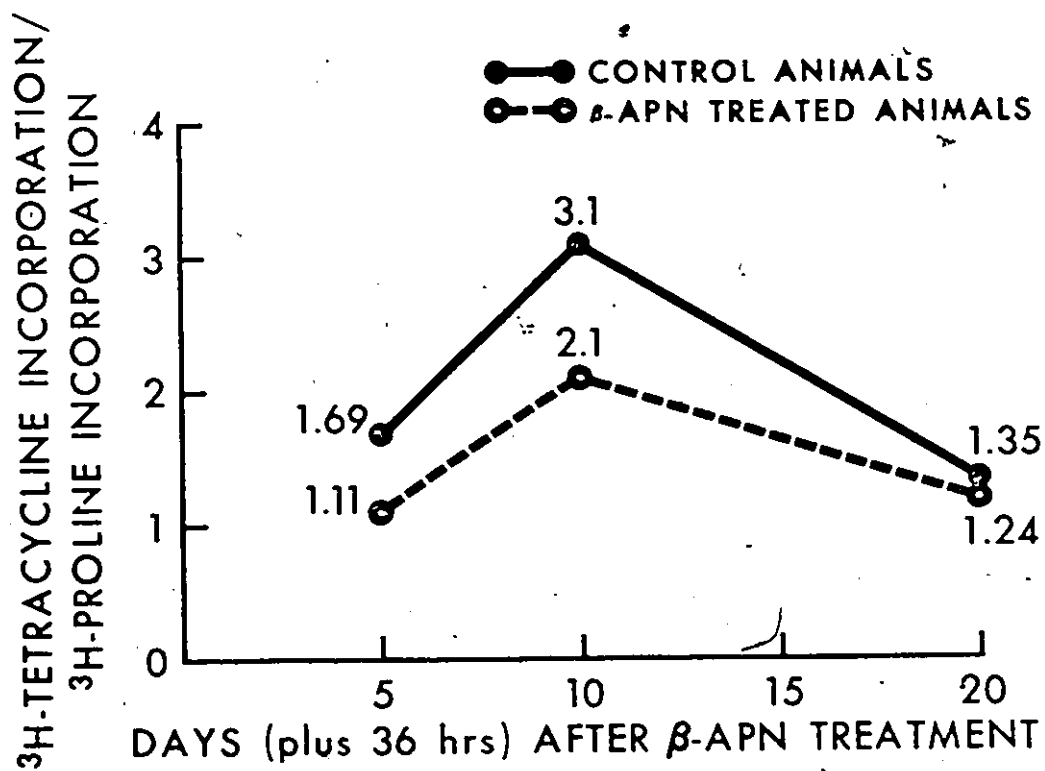
3.2.3

^3H -PROLINE ACTIVITY REMAINING IN RAT
(TREATED WITH β -APN FOR 3 WEEKS) TIBIA
AT DIFFERENT TIMES (HRS) AFTER DOSING



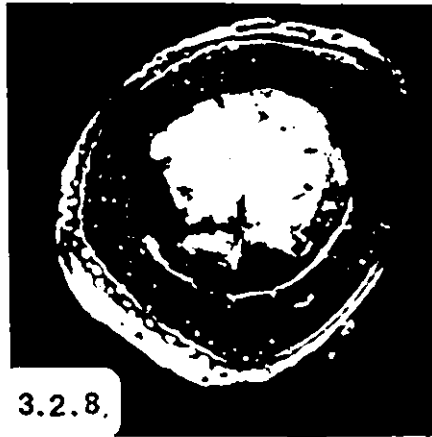
3.2.4

^3H -TETRACYCLINE REMAINING AT 36 HRS. AFTER DOSING

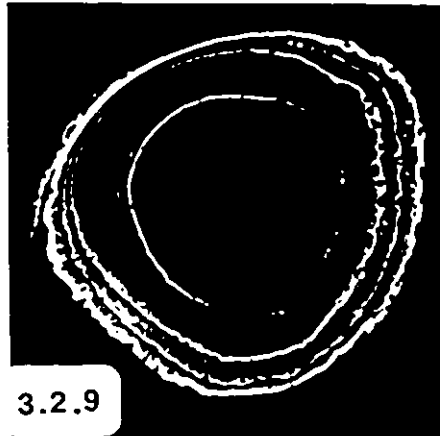




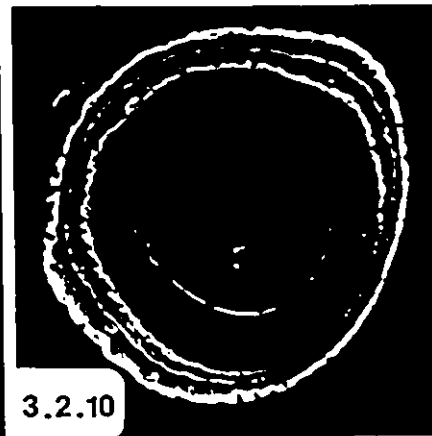
3.2.7



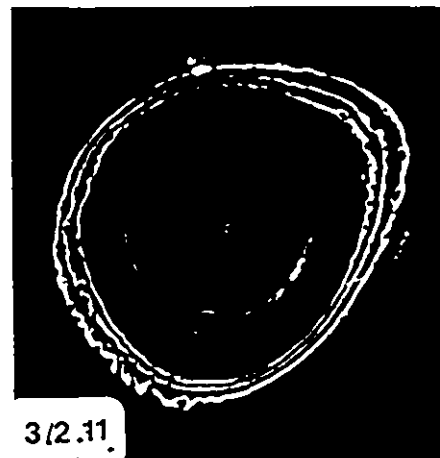
3.2.8



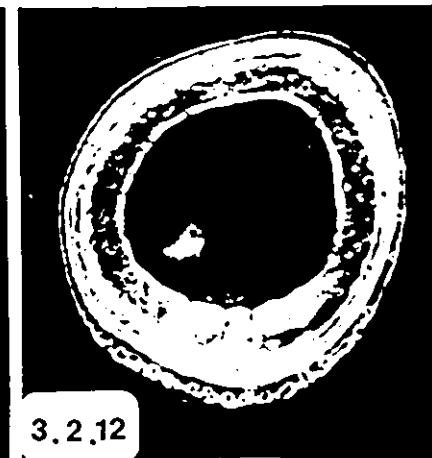
3.2.9



3.2.10



3.2.11



3.2.12

Figures 3.2.7, 9 and 11 are from the control rats while Figures 3.2.8, 10 and 12 are from the rats treated with B-APN. Figures 3.2.7 and 8 show the bands from cold tetracycline administration of 0 and 5 days. Figures 3.2.9 and 10 show three bands from 0, 5 and 10 days. Figures 3.2.11 and 12 show four labels from cold tetracycline administered at 0, 5, 10 and 20 days. X20.

3.3 EFFECTS OF B-AMINOPROPIONITRILE ON BONE MATRIX FORMATION AND ITS MINERALIZATION IN CHICKS

3.3.1 Rationale for Study on Chicks

In the rat diaphyseal growth, the bone matrix formation not only takes place on the periosteal surface but also at certain locations on endosteal surface. In addition it was found rather impossible to take into account the bone resorption. The growth of the diaphyseal bone in chicks, presents a simpler system where the bone growth takes place mainly on the periosteal surface and the resorption on the endosteal surface (Jande, 1971). It is thus easy to follow the location of tetracycline fluorescent bands for the experiments of longer duration. Also, a species difference in sensitivity to B-APN has been suggested by Barrow et al., (1974).

This study was conducted in two phases. In the preliminary phase, to establish a dose of B-APN which will not inhibit the general body growth, two week old white Leghorn male chicks were fed diets containing B-APN (0.05%, 0.10% or 0.25%) in three separate groups.

The chicks on the two higher doses showed a severe retardation of body weight gain and tibial length after one week. On this basis, for further experiments, only chicks on the diet containing 0.05% B-APN were used. The controls were fed a chick biostartena diet (Purina Co., Whitby, Ontario).

TABLE 3.3.1.

BODY WEIGHT AND TIBIAL LENGTH OF CONTROLS AND B-APN TREATED CHICKS

TIME*	PARAMETER	CONTROL ANIMALS	B-APN TREATED ANIMALS	P ^a
7 days plus 36 hours	1. Body Weight	211.00 ± 15.84 ^b gms	209.00 ± 7.97 gms	NS ^c
	2. Tibial Length	5.290 ± 0.098 cm	5.457 ± 0.060 cm	NS
14 days plus 36 hours	1. Body Weight	314.40 ± 14.11 gms	313.60 ± 11.72 gms	NS
	2. Tibial Length	6.21 ± 0.090 cm	6.17 ± 0.050 cm	NS
21 days plus 36 hours	1. Body Weight	465.80 ± 5.17 gms	467.20 ± 5.58 gms	NS
	2. Tibial Length	7.63 ± 0.0674 cms	7.662 ± 0.037 cms	NS

All data are based on results from at least 6 chicks.

- a. Probability estimated by Students' T-Test
- b. Mean + S.E.
- c. Not significant at 0.05 probability level.

* Days denote the length of B-APN treatment and the hours denote the time after dosing with the labels. Thus the total time of treatment equals days plus 36 hours.

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3.3.2 Effects of B-aminopropionitrile on General Body Growth and Tibial Length

For evaluating the effect of B-APN (0.05% of diet) on general body growth and that of bones at organ level the body weight and tibial lengths were recorded at 7, 14, and 21 days of the experiment.

The changes in body weight and tibial lengths, during the experiment, of controls and B-APN fed chicks are shown in Table 3.3.1. The values represent the mean \pm standard errors of the means for data from at least six chicks. The body weight of the control chicks was 211.00 \pm 15.84 gms, 314.40 \pm 14.11 gms and 465.40 \pm 5.17 gms at 7, 14 and 21 days respectively. The body weight of the B-APN fed chicks was 209.00 \pm 7.97 gms, 313.60 \pm 11.72 gms and 467.20 \pm 5.58 gms at similar intervals as mentioned for the controls. The tibial lengths of the controls were 5.290 \pm 0.098 cm, 6.21 \pm 0.090 cm and 7.63 \pm 0.0674 cm at 7, 14 and 21 days respectively while that of B-APN fed chicks were 5.457 \pm 0.060 cm, 6.17 \pm 0.050 cm and 7.662 \pm 0.037 at similar intervals.

3.3.3 Effects on Bone Ash Contents

To evaluate the effects of B-APN on mineral contents of the bones, the bone ash contents were determined according to a method of Rosenquist et al., (1977) as described in the materials and methods.

The bone ash contents of the controls and B-APN treated

TABLE 3.3.2.

BONE ASH CONTENTS AND SERUM CALCIUM OF CONTROLS AND B-APN TREATED CHICKS

TIME *	PARAMETER	CONTROL ANIMALS	B-APN TREATED ANIMALS	P ^a
7 days plus 36 hours	1. Bone Ash Content	63.37 ± 0.41 ^b %	60.57 ± 0.015 %	<0.01
	2. Serum Calcium	10.02 ± 0.13 mg%	9.76 ± 0.31 mg%	NS ^c
14 days plus 36 hours	1. Bone Ash Content	62.62 ± 0.34.%	60.54 ± 0.68 %	<0.05
	2. Serum Calcium	9.82 ± 0.14 mg%	9.69 ± 0.30 mg%	NS
21 days plus 36 hours	1. Bone Ash Content	61.79 ± 0.58 %	59.49 ± 0.0032 %	<0.02
	2. Serum Calcium	10.12 ± 0.14 mg%	10.11 ± 0.09 mg%	NS

All data are based on observations from at least 6 chicks.

- a. Probability estimated by Students' T-Test
- b. Mean ± S.E.
- c. Not significant at 0.05 probability level.

* Days denote the length of B-APN treatment and the hours denote the time after dosing with the labels. Thus the total time of treatment equals days plus 36 hours.

chicks are shown in Table 3.3.2. The values represent mean \pm standard error of the means for data from six chicks in each group. The bone ash contents of the controls were 63.37%, 62.62% and 61.79% while those of B-APN fed chicks were 60.57%, 60.54% and 59.49% at 7, 14 and 21 days respectively.

3.3.4 Effects on Serum Calcium Level

The serum calcium level is an indicator of stability of the calcium homeostasis. The B-APN effect on this parameter was evaluated by measuring the serum calcium level as described in an earlier section. The data again represents observations from six chicks in each group. The serum calcium level of the controls was 10.02 ± 0.13 mg%, 9.82 ± 0.14 mg% and 10.12 ± 0.14 mg% while that of the B-APN fed chicks was 9.76 ± 0.31 mg%, 9.69 ± 0.30 mg% and 10.11 ± 0.09 mg% at 7, 14 and 21 days respectively.

3.3.5 Microradiography

Figures 3.3.1, 2, 3, 4, 5 and 6 are the microradiographs of cross sections of tibial diaphyses of the controls and B-APN treated chicks at 7, 14 and 21 days.

The size of the lacunar cavities and radiodensity of the bone appears similar in both groups. In addition to evaluating the radiodensity of bone cross sections another two parameters were measured from the microradiographs.

1. Total cortical cross section area, and 2. total medullary cross section area. These two parameters, provide information about the total bone formed and also about any

TABLE 3.3.3.

AREA OF CORTICAL BONE AND AREA OF THE MARROW OF CONTROLS AND B-APN TREATED CHICKS

TIME *	PARAMETER	CONTROL ANIMALS	B-APN TREATED ANIMALS	P ^a
7 days plus 36 hours	1. Area of Cortical Bone	5.246 ± 0.3025 mm ²	5.531 ± 0.190 mm ²	NS ^c
	2. Area of the marrow	3.853 ± 0.155 mm ²	4.055 ± 0.135 mm ²	NS
14 days plus 36 hours	1. Area of Cortical Bone	5.529 ± 0.381 mm ²	5.316 ± 0.316 mm ²	NS
	2. Area of the marrow	3.940 ± 0.218 mm ²	4.045 ± 0.186 mm ²	NS
21 days plus 36 hours	1. Area of Cortical Bone	6.559 ± 0.387 mm ²	6.746 ± 0.234 mm ²	NS
	2. Area of the marrow	6.264 ± 0.194 mm ²	5.881 ± 0.369 mm ²	NS

All data based upon observations from at least 5 chicks and 5 readings were taken from each section.

- a. Probability estimated by Student's T-Test
- b. Mean ± S.E.
- c. Not significant at 0.05 probability level.

* Days denote the length of B-APN treatment and the hours denote the time after dosing with the labels. Thus the total time of treatment equals days plus 36 hours.

differences in resorption between two groups at the endosteum level.

(i) Effects on Total Cross Section Cortical and Medullary Areas

The total cross section cortical area and total cross section medullary area in the controls and B-APN fed chicks are shown in Table 3.3.3. The values represent mean \pm SE of the means for data from at least 5 chicks in each group.

The total cross section cortical area in the controls was 5.246 \pm 0.3025 mm², 5.529 \pm 0.381 mm² and 6.559 \pm 0.387 mm² at 7, 14 and 21 days while similar measurements from the B-APN fed chicks gave values of 5.531 \pm 0.190 mm², 5.316 \pm 0.316 mm² and 6.746 \pm 0.234 mm² at similar intervals as described for the controls.

The total cross section medullary area in controls was 3.853 \pm 0.155 mm², 3.940 \pm 0.218 mm² and 6.264 \pm 0.194 mm² while in the B-APN fed chicks it was 4.055 \pm 0.135 mm², 4.045 \pm 0.186 mm² and 5.881 \pm 0.369 mm² at 7, 14 and 21 days respectively.

3.3.6 Fluorescent Microscopy

The technique of fluorescent microscopic labelling was used to determine the linear bone apposition in the controls and B-APN treated chicks during the experiments. The Figures 3.3.7 and 8 show the tetracycline labels from the injections at 0, 7, 14 and 21 days in the controls and B-APN fed chicks respectively.

TABLE 3.3.4.

WIDTH OF BONE FORMED BETWEEN EACH TIME INTERVAL IN CONTROL AND B-APN TREATED CHICKS^a

TIME	LINEAR BONE APPPOSITION BETWEEN INTERVALS	P ^b
0-7 days Control	353.06 ± 9.93 ^c μm	NS ^d
B-APN Treated	358.33 ± 8.74 μm	
7-14 days Control	187.88 ± 4.62 μm	NS
B-APN Treated	187.44 ± 4.68 μm	
14-21 days Control	161.51 ± 5.82 μm	NS
B-APN Treated	158.22 ± 3.36 μm	

All data are based upon observations from at least 5 chicks and 5 readings were taken from each section.

- a. Diet 0.05% B-APN
- b. probability estimated by Students' T-Test
- c. Mean ± S.E.
- d. Not significant at 0.05 probability level.

(i) Effects on Linear Bone Apposition

The linear bone apposition measurements were made from the outer edge of preceeding label to inner edge of the ~~past~~ current band. The measurements were taken from at least 5 sections taken from 5 different chicks in each group.

The linear bone apposition in the controls was 353.06 ± 9.93 μm , 187.88 ± 4.62 μm and 161.51 ± 5.82 μm at 7, 14 and 21 days interval. The linear bone apposition in the B-APN fed chicks was 358.33 ± 8.74 μm , 187.44 ± 4.68 μm and 158.22 ± 3.36 μm at 7, 14 and 21 days interval respectively.

3.3.7 Effects on Incorporation of ^3H -Proline and ^3H -Tetracycline

Since we had established in rats, the time of sacrifice after radioisotopic administration and other parameters to evaluate the bone matrix formation and its mineralization with the help of ^3H -proline and ^3H -tetracycline incorporation measurements, a similar time was used for the study on chicks.

Thirty-six chicks were divided in two groups. The controls were fed normal chick food (as mentioned earlier) and the second group was fed a diet containing B-APN (0.05%) for three weeks.

The CPM/100 mg wet tibial weight were calculated from the equal lengths of tibial diaphyses of 6 chicks in each group, sacrificed thirty-six hours after the administration of

TAB. 3.3.5.

³H-TETRACYCLINE AND ³H-PROLINE INCORPORATION IN TIBIAS OF CONTROLS AND B-APN TREATED CHICKS

TIME*	PARAMETER	CONTROL ANIMALS	B-APN TREATED ANIMALS	P ^a
7 days plus 36 hours	1. ³ H-tetracycline incorporation	4.0767 ± 0.1080 %	3.433 ± 0.0681 %	<0.01
	2. ³ H-proline incorporation	1.2452 ± 0.0845 %	1.2810 ± 0.0944 %	NS
14 days plus 36 hours	1. ³ H-tetracycline incorporation	0.2418 ± 0.0020 %	0.2184 ± 0.0057 %	<0.02
	2. ³ H-proline incorporation	0.1808 ± 0.0044 %	0.1815 ± 0.0039 %	NS
21 days plus 36 hours	1. ³ H-tetracycline incorporation	0.2079 ± 0.006 %	0.1536 ± .005 %	<0.001
	2. ³ H-proline incorporation	0.1119 ± 0.0017 %	0.1145 ± 0.0032 %	NS

All data is based on results from at least 6 chicks.

a. Probability estimated by students' T-Test

b. Mean ± S.E.

c. Not statistically significant of 0.05 probability level.

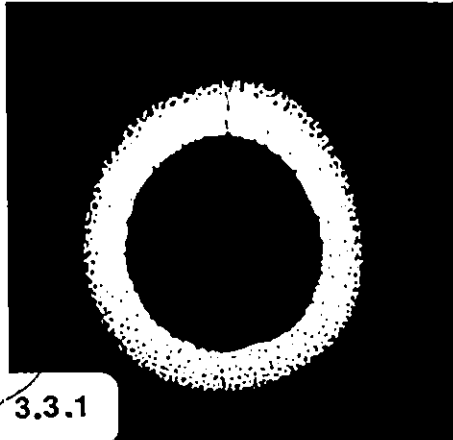
* Days denote the length of B-APN treatment and the hours denote the time after dosing with the labels. Thus total time of treatment equals days plus 36 hours.

^3H -proline (10 uCi/60 gm body weight) or with ^3H -tetracycline (5 uCi/100 gm body weight) at intervals of 7, 14 and 21 days, by preparing their bones for liquid scintillation counting as described earlier.

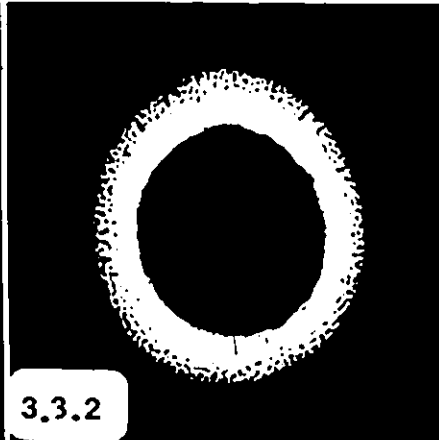
These results are shown in Table 3.3.5. The values represent the mean \pm standard error of the means for the data from at least 6 chicks.

The incorporation of ^3H -proline in the controls was 1.2452 \pm 0.0845%, 0.1808 \pm 0.004% and 0.1119 \pm 0.0017% while that in the bones of B-APN treated chicks was 1.2810 \pm 0.0944%, 0.1815 \pm 0.0039%, 0.1145 \pm 0.0032% at 7, 14 and 21 days respectively.

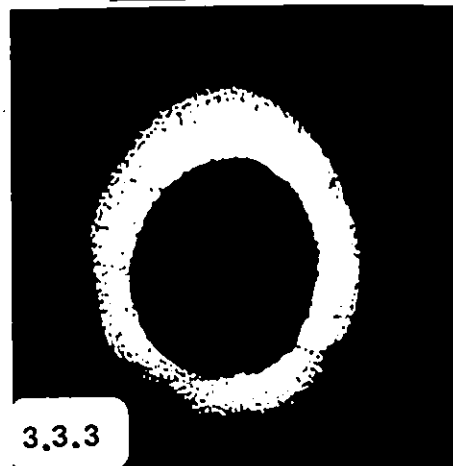
The incorporation of ^3H -tetracycline, calculated as percentage of initial dose administered is also shown in Table 3.2.5. It was 4.0767 \pm 0.1080%, 0.2418 \pm 0.002% and 0.2079 \pm 0.006% in the controls while 3.433 \pm 0.0681%, 0.2184 \pm 0.0057% and 0.1536 \pm 0.005% in the B-APN fed chicks at 7, 14 and 21 days respectively.



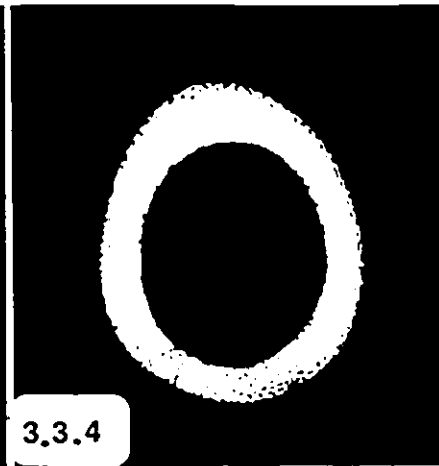
3.3.1



3.3.2



3.3.3



3.3.4

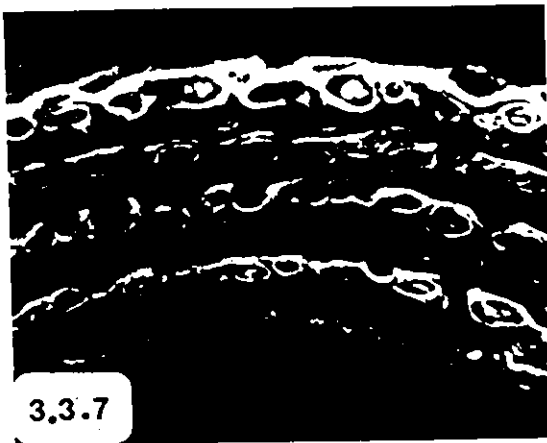


3.3.5

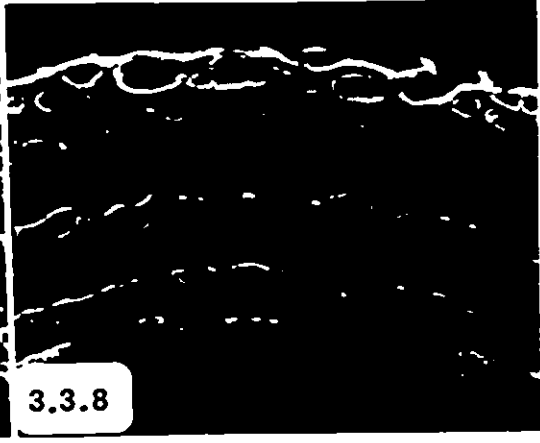


3.3.6

Figures 3.3.1, 3 and 5 are microradiographs of the tibial cross sections from the control chicks while Figures 3.3.2, 4 and 6 are the microradiographs of B-APN treated chick tibial cross sections. X15



3.3.7



3.3.8

Figure 3.3.7. A part of the control chick tibial cross section under UV light administered cold tetracycline at 0, 7, 14 and 21 days. The band present at the top of picture represent the injection at 21 days. X75.

Figure 3.3.8. A UV photomicrograph similar to Figure 3.3.7 but from the B-APN fed chicks. X75.

TABLE 3.4.1.

BODY WEIGHT, TIBIAL LENGTH AND BONE ASH CONTENT OF CONTROLS AND B-APN TREATED CHICK EMBRYOS

PARAMETERS	CONTROL ANIMALS	B-APN TREATED	P ^c
1. Body Weight	3.4762 ± 0.247 ^a gm.	3.6256 ± 0.076 gm	NS ^b
2. Tibial Length	6.08 ± 0.01 cm.	5.96 ± 0.02 cm	NS
3. Bone Ash Content	18.717 ± 1.0798 %	15.2238 ± 1.0638 %	< 0.05

All data are based on observations from at least 5 embryos.

a. Mean + S.E.

b. Not significant at 0.05 probability level

c. Probability estimated by Student's T-test.

3.4 EFFECTS OF B-AMINOPROPIONITRILE ON BONE MATRIX FORMATION AND ITS MINERALIZATION IN CHICK EMBRYOS

The eggs from White Leghorn hens were used in this investigation. On the 7th day of incubation the B-APN (100 ul of 0.1% aqueous solution) was injected in the air chambers as explained in the materials and methods. The controls were injected Sod. fumarate in similar quantities. The administration continued until day 11.

At the time of sacrifice, day 12, there were no mortalities in the controls as well as in the eggs injected with B-APN.

3.4.1 Effects on General Body Growth and Tibial Length

The body weight of the embryos from the controls was 3.4762 ± 0.247 gm while that of B-APN treated embryos was 3.6256 ± 0.076 gm. These data are shown in Table 3.4.1. The values in the table represent mean \pm standard error of the means and these values are based on the observations from at least 5 embryos in each group.

Tibias from the embryos which were injected B-APN were slightly bent in the middle (Fig. 3.4.1). The incidence of deformed tibia was present in one hundred percent of the embryos injected with B-APN.

The length of the tibiae in the controls was 6.08 ± 0.01 mm while that in the B-APN fed embryos was 5.96 ± 0.02 mm. These data is also shown in Table 3.4.1.

TABLE 3.4.2.

3H-PROLINE AND 3H-TETRACYCLINE INCORPORATION IN BONES OF CONTROLS AND B-APN TREATED CHICK EMBRYOS

PARAMETER	CONTROL	B-APN TREATED	P ^c
1. 3H-Proline Incorporation	0.2612 ± 0.172%	0.2600 ± 0.055%	NS ^b
2. 3H-Tetracycline Incorporation	0.1905 ± 0.014%	0.1021 ± 0.0056%	< 0.01

All data are based on observations from at least 5 embryos.

a. Mean + S.E.

b. Probability estimated by Students' T-Test

c. Not significant at 0.05 probability level.

3.4.2 Effects on Bone Ash Contents

The bone ash contents of the tibias from the controls and B-APN treated embryos are shown in Table 3.4.1. The bone ash contents in the controls were $18.717 \pm 1.0798\%$ of the net tibial weight while in B-APN injected embryos it was $15.2238 \pm 1.0638\%$ of the net weight.

3.4.3 Effects on Incorporation of ^3H -Proline and

^3H -Tetracycline

To see the effect of B-APN on the bone matrix formation and its mineralization the incorporation of ^3H -proline and ^3H -tetracycline was analysed in the mineralized portions of embryonic tibias. The CPM/5 mg wet tibial weight from the mineralized portions of embryonic tibias of chick embryos, injected either ^3H -proline (1 uCi/embryo) or with ^3H -tetracycline (1 uCi/embryo) on 11th day and sacrificed thirty-six hours later, were calculated. The incorporation of both isotopes was calculated as the percentage of initial dose administered/5 mg tibial weight and is shown in Table 3.4.2. The values represent means \pm standard error of the means for the data from at least 5 embryos in each group.

The incorporation of ^3H -proline in the controls was $0.2612 \pm 0.172\%$ while in the B-APN treated embryos it was $0.2600 \pm 0.055\%$. The incorporation of ^3H -tetracycline in the controls was $0.1905 \pm 0.014\%$ while it was $0.1021 \pm 0.0056\%$ in the B-APN treated embryos.

TABLE 3.4.3.

ACTIVITIES OF ALKALINE PHOSPHATASE, Ca^{++} , Mg^{++} -ATPase, ACID PHOSPHATASE AND Na^+ , K^+ -ATPase IN BONES OF CONTROLS AND B-APN TREATED CHICK EMBRYOS.

PARAMETER	CONTROL	B-APN TREATED	P _c
Alkaline Phosphatase	308.40 ± 23.90 ^a	119.20 ± 3.3	< 0.001
	uM P-nitrophenol released/mg/protein/hr.		
Ca^{++} , Mg^{++} -ATPase	84.02 ± 0.58	12.31 ± 0.30	< 0.001
	uM Pi-released/mg. protein/hr.		
Acid Phosphatase	3555.00 ± 494.8	4965.00 ± 204.10	< 0.05
	uM P-nitrophenol released/mg. protein/hr.		
Na^+ , K^+ -ATPase	36.94 ± 7.1	32.60 ± 2.98	NS ^b
	uM Pi-released/mg. protein/hr.		

All data are based on observations from at least 5 embryos.

a. Mean ± S.E.

b. Not significant at 0.05 probability level

c. Probability estimated by Student's T-Test.

3.4.4 Effect Of B-APN On Certain Enzymes In Bone and Liver

Tissues

(i) Biochemical Assays

The activity of alkaline phosphatase, Ca^{++} -ATPase, Na^+ , K^+ -ATPase, in mineralized portions of embryonic tibias and embryonic liver tissue, was measured in membrane fractions isolated from the tissues as described in the materials and methods. The subcellular particles isolated from the embryonic bones and liver, as described in materials and methods, were used to measure the activity of the acid phosphatase.

The activities of alkaline phosphatase, Ca^{++} -ATPase, Na^+ , K^+ -ATPase and acid phosphatase in bones of the controls and B-APN treated embryos are shown in Table 3.4.3. The values represent means \pm SE of the means for data from at least 5 experiments.

The alkaline phosphatase activity expressed as μM p-nitrophenol released/mg protein/hour was 308.40 ± 23.90 in the controls and 119.20 ± 3.3 in the bones of B-APN treated embryos.

The activity of Ca^{++} -ATPase expressed as μM P_i -released/mg protein/hour in the controls was 84.02 ± 0.58 while it was 12.31 ± 0.30 in the B-APN treated embryonic bones.

The acid phosphatase activity is expressed as μM p-nitrophenol/mg protein/hour. In the controls it was 3555.00 ± 494.8 while in the B-APN treated embryos it was 4965.00 ± 204.10 .

TABLE 3.4.4.

ACTIVITIES OF ALKALINE PHOSPHATASE, Ca^{++} , Mg^{++} -ATPase, ACID PHOSPHATASE, Na^+ , K^+ -ATPase IN LIVER
TISSUE OF CONTROL AND B-APN TREATED CHICK EMBRYOS.

PARAMETER	CONTROL	B-APN TREATED	P ^c
1. Alkaline Phosphatase uM P-nitrophenol released/mg. protein/hr.	506.00 + 44.77 ^a	560.00 ± 40.43	NS ^b
2. Ca^{++} , Mg^{++} -ATPase uM Pi-released/mg. protein/hr.	106.20 + 7.28	104.88 ± 7.13	NS
3. Acid Phosphatase uM P-nitrophenol released/mg. protein/hr.	296.00 + 307.65	2837.00 ± 114.47	NS
4. Na^+ , K^+ -ATPase uM Pi-released/mg. protein/hr.	49.03 + 5.3	51.23 ± 6.6	NS

All data are based on observation from at least 5 embryos.

- a. Mean + S.E.
- b. Not significant at 0.05 probability level
- c. Probability estimated by Students' T-Test.

Na^+ , K^+ -ATPase activity, expressed as $\mu\text{M Pi-released/mg protein/hour}$, in the controls was 36.94 ± 7.1 while in the B-APN treated embryo bones it was 32.60 ± 2.98 .

The activities of alkaline phosphatase, Ca^{++} -ATPase, Na^+ , K^+ -ATPase and acid phosphatase in liver tissue of the controls and B-APN treated embryos are shown in Table 3.4.4. The values represent \pm standard error of the means for data from at least 5 experiments.

The alkaline phosphatase activity was $506.00 \pm 44.77 \mu\text{M p-nitrophenol/mg protein/hour}$ in the controls while in the B-APN treated embryonic liver it was $560.00 \pm 40.43 \mu\text{M p-nitrophenol released/mg protein/hour}$.

Ca^{++} -ATPase activity in the controls was $106.20 \pm 7.28 \mu\text{M Pi released/mg protein/hour}$ while it was $104.88 \pm 7.13 \mu\text{M Pi released/mg protein/hour}$ in the B-APN treated embryo liver tissue.

The acid phosphatase activity expressed as $\mu\text{M p-nitrophenol released/mg protein/hour}$ in the controls was 2960.00 ± 307.65 while it was 2837.00 ± 114.47 in the B-APN treated embryonic liver tissue.

Na^+ , K^+ -ATPase activity in the controls was $49.03 \pm 5.3 \mu\text{M Pi released/mg protein/hour}$. In B-APN treated chick embryonic tissue it was $51.23 \pm 6.6 \mu\text{M Pi released/mg protein/hour}$.

(ii) Histochemistry

In order to see the locale of the enzyme alkaline phosphatase and acid phosphatase, these enzymes were localized histochemically. These two enzymes were also localized at ultrastructural level.

(a) Alkaline Phosphatase

(i) Light Microscopy

For localization of alkaline phosphatase the technique of Burstone (1958) was used. The incubation was carried out for 30 and 60 minutes and the photomicrographs represent sections incubated for 30 minutes only.

The reaction product was present mainly at the periosteal surface with little or none at the endosteal surface (Fig. 3.4.2). The osteogenic cells and osteoblasts showed the reaction while the outer layers of cells of periosteal envelope were negative (Fig. 3.4.2). The osteogenic cells and osteoblasts showed reaction product on their plasma membranes which, under high power, appeared as dark material on the cell membranes (Fig. 3.4.3). The cells lining the surfaces of newly formed bone trabeculae also showed a positive reaction on their membranes.

The control sections from rat kidney, fixed and further prepared in a manner similar to the bone samples, showed the reaction product on the striated border of the cells of proximal tubules of the kidney (Fig. 3.4.4).

(ii) Electronmicroscopy

The localization of alkaline phosphatase at ultrastructural level was done according to method described by Bernard (1978).

An incubation time of 45 minutes was found to give very satisfactory results.

The electronmicroscopy confirmed our observations of light microscopy. The cells lining the inner layers of periosteal envelope showed an electron dense granular reaction product on their plasma membranes (Fig. 3.4.5). The reaction product was not only present on plasma membranes of cells immediately next to the osteoid surface, but was seen on the plasma membranes of many layers of cells (Fig. 3.4.5). In fact quite often, the cell membranes towards pre-osseous zone did not show any reaction product of the alkaline phosphatase activity (Fig. 3.4.5). The osteoblasts forming the bone membrane over the preosseous zone showed reaction product on the plasma membranes where the cell membranes were apposed to each other (Fig. 3.4.6).

The cells of the outer layers of the periosteum, believed to be fibroblasts, did not show any reaction product (Figs. 3.4.5 and 3.4.7).

The matrix vesicles, in the preosseous zone of the bone matrix, were positive for alkaline phosphatase reaction (Fig. 3.4.8). The reaction product was not only present on their membranes but their interior also showed an electron dense granular reaction product (Fig. 3.4.9).

In rat kidney sections the reaction product was present in the striated border of the cells of the proximal convoluted tubules (Fig. 3.4.10).

The cells of the outer layers of the periosteum, believed to be fibroblasts, did not show any reaction product (Fig. 3.4.5).

(b) Acid Phosphatase

(i) Light Microscopy

The acid phosphatase was localized according to method of Burstone (1958). The only reaction product that could be observed in cross sections of embryonic tibia, was present at the endosteal surface (Fig. 3.4.11). The cells which showed a positive reaction were very large. The overwhelming presence of reaction product inside those cells made it difficult to recognize the cellular morphology (Fig. 3.4.11). The osteoblasts, chondrocytes and the fibroblasts of the periosteum did not show any reaction for acid phosphatase whatsoever (Fig. 3.4.11).

In kidney sections, kept as control for the histochemical technique, the acid phosphatase activity was present intracellularly in the cells of proximal as well as distal convoluted tubules (Fig. 3.4.12). The reaction product appeared as discrete vesicular structures presumably lysosomes.

(ii) Electron microscopy

For cytochemical localization of acid phosphatase the method of Gomori (1943) modified by Barka and Anderson (1963) were used.

At the ultrastructural level the acid phosphatase activity, indicated by the presence of electron dense precipitate, was localized in the dense vesicular structures presumably lysosomes in the osteoblasts (Fig. 3.4.13, 14). There was some fibrillar material present in these dense bodies (Figs. 3.4.15, 16) and these findings are similar to the ones of Jande and Grosso (1975). In some osteoblasts small amount of acid phosphatase reaction was also present in association with Golgi saccules and vesicles (Fig. 3.4.16).



Control



3.4.1

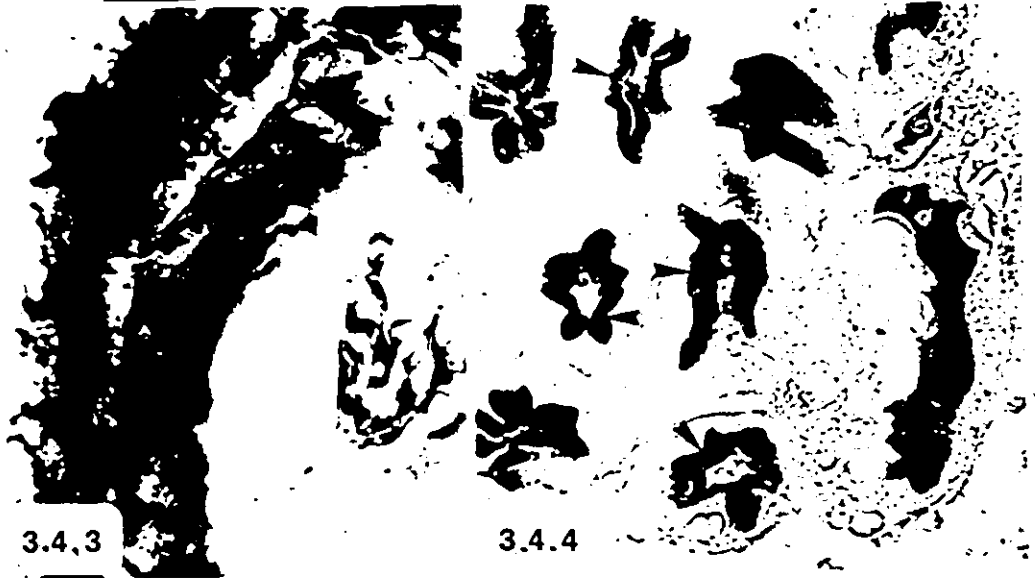
B-APN



Fig. 3.4.1. Photomicrograph of tibias from the controls and B-APN treated chick embryos. Note the deformity in B-APN treated tibias X 6.



3.4.2



3.4.3

3.4.4

All photomicrographs from the control chick embryos.

Fig. 3.4.2. A low magnification photomicrograph showing a part of the chick embryo tibial cross section, incubated (30 minutes) for alkaline phosphatase. The dark reaction product is seen mainly in the inner periosteum (arrows) and on the surface of peripheral trabeculae (arrow heads). The outer fibrillar layers of the periosteum (asterisks) are negative X 160.

Fig. 3.4.3. A higher magnification photomicrograph of a trabecula (bt) from a section similar to that of Fig. 3.4.1. The reaction product is seen on the osteoblast surfaces (arrow heads) and in the immature bone (arrow) X 720.

Fig. 3.4.4. A photomicrograph of a rat kidney cortex section, incubated similar to that of Fig. 3.4.1. Note that the reaction product is only in the striated border of the cells of proximal convoluted tubules (arrow heads) X 1440.



Per

Pre Os

3.4.5

Fig. 3.4.5. A section passing through the zone of initial mineralization and osteoblasts (OB) lying adjacent to preosseous tissue. (Pre Os.). Material was incubated for alkaline phosphatase reaction (Gomori Method) for 30 minutes. Dark electron dense reaction product is seen on cellular membranes (arrow heads) and in the pre osseous tissue (arrow). Also seen in pre osseous tissue are collagen fibrils (Cf) with their characteristic banding pattern. At upper right side of the picture is periosteum (Periost.). Note that cells in this region do not have any reaction product associated with their plasma membranes X 16,100. Normal chick material.



34.6

Fig. 3.4.6. Parts of three osteoblasts (OB) forming the bone membrane. The material was incubated for alkaline phosphatase. The reaction product is associated with plasma membranes (arrows) and even at the surface where osteoblast plasma membrane is apposed to the membrane of adjacent osteoblast X 82,080. Normal chick material.

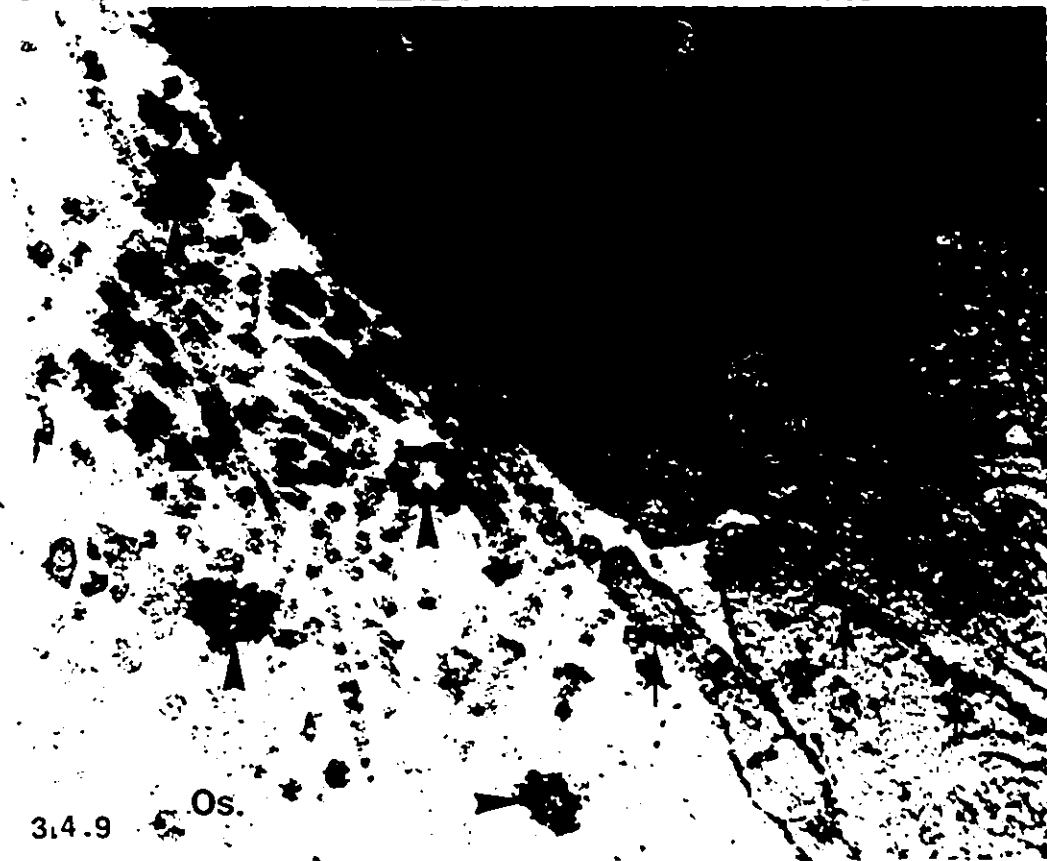


Periost.

Pre Os.

3.4,7

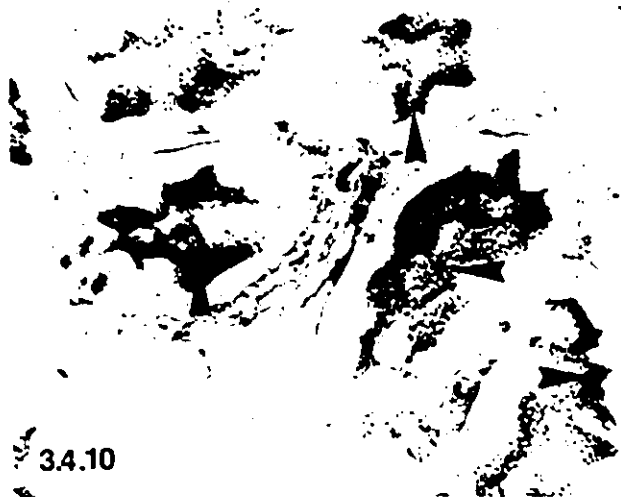
Fig. 3.4.7. High power electronmicrograph of an area adjacent to zone of the initial mineralization from chick embryonic tibia incubated for alkaline phosphatase (Gomori method). Note the dark reaction product on osteoblastic plasma membranes and in the pre osseous (Pre Os.) tissue (arrow heads) X 27,360. Normal chick material.



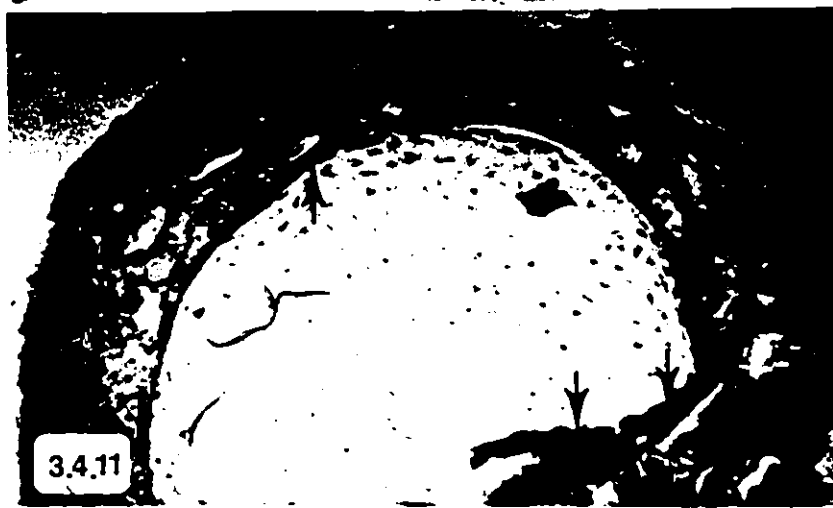
Both electronmicrographs from the controls.

Fig. 3.4.8. An electronmicrograph from part of a cross section of embryonic tibia incubated for alkaline phosphatase. Note the electron dense reaction product (arrows) on the plasma membranes of osteoblasts (OB) as well as (arrow heads) in the osteoid (Os) X 16,100.

Fig. 3.4.9. A higher magnification electronmicrograph of the area marked in Fig. 3.4.8. The electron dense reaction product (arrows) is seen clearly on the plasma membranes of osteoblasts (Ob). In the osteoid (Os) the reaction product is present mainly in relation to matrix vesicles (arrow heads) X 51,300.



3.4.10



3.4.11



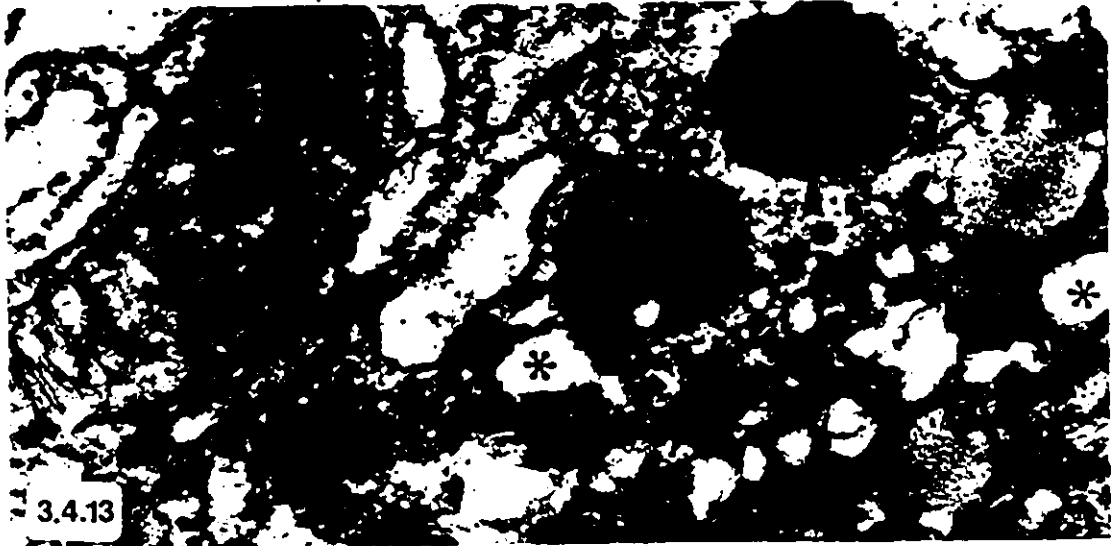
3.4.12

All figures from the control material.

Fig. 3.4.10. A photomicrograph of section of the cortex of kidney incubated for alkaline phosphatase reaction (Gomori method). Arrow heads point toward the dark granular reaction product in striated border of proximal convoluted tubules only X 150.

Fig. 3.4.11. A photomicrograph of the cross section of chick embryonic tibia incubated for acid phosphatase (Burstone's method). Dark reaction product is seen in the osteoclasts on the endosteal surface (arrows) X 110.

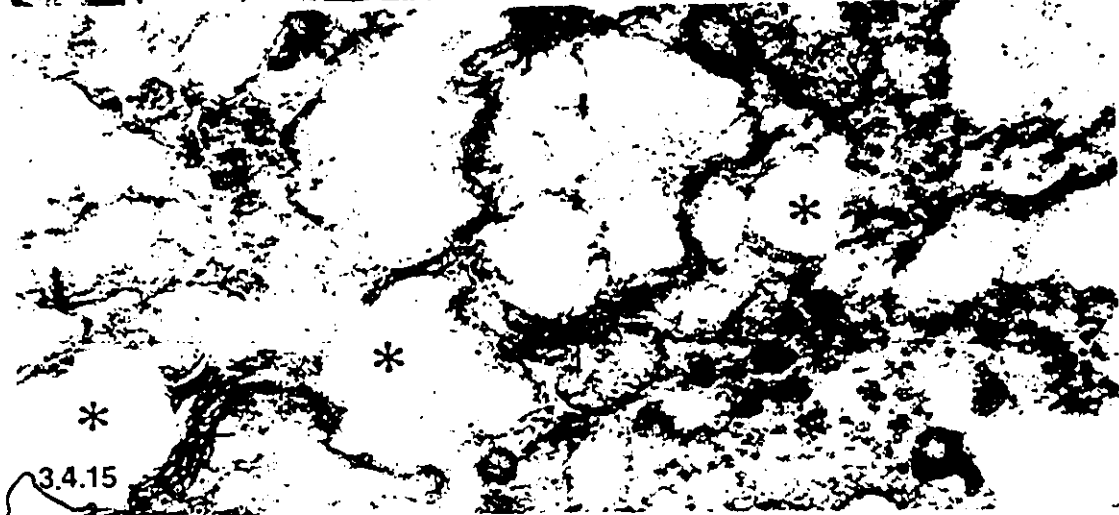
Fig. 3.4.12. A photomicrograph of section from the cortex of kidney incubated for acid phosphatase (Gomori method). Note the dark reaction product (arrow heads) intra cellularly in the cells of convoluted tubules. There is no reaction product present in the striated border of proximal tubules X 150.



3.4.13



3.4.14



3.4.15

All figures from the control material.

Fig. 3.4.13. An electronmicrograph of the section from chick embryonic tibia incubated (30 minutes) for acid phosphatase. Distended ends of Golgi saccules (asterisk) are seen and dark granular reaction product is seen in association with Golgi saccules (small arrows) and inside the typical lysosome (arrow head). Some fibrillar material is also seen in another lysosome (big arrow head) X 82,080.

Fig. 3.4.14. A similar section as seen in Fig. 3.4.13. Dark reaction product is seen in a lysosome (arrow head). Also seen is some fibrillar material inside the lysosome X 82,080.

Fig. 3.4.15. A high power electronmicrograph of Golgi region (asterisk) seen in Fig. 3.4.13. Note the dark reaction product on the walls of distended ends of Golgi saccules (arrows) X 82,080.

3.5 EFFECTS OF B-AMINOPROPIONITRILE ON BONE MEMBRANE ON THE

PERIOSTEAL SURFACE OF CHICK EMBRYONIC BONES

A single layer of osteoblasts lining all the formative surfaces of bone has been described as "bone membrane" (Talmage, 1969, 1970, Talmage et al., 1970 Tonna, 1978).

Electronmicroscopy

In our study of chick embryonic bones, a similar arrangement of osteoblasts was observed in the controls (Fig. 3.5.1) as well as in the bones of B-APN treated embryos (Fig. 3.5.4). In 12 days old chick embryos, osteoblasts and preosteoblasts lined the periosteal surfaces in the controls as well as B-APN treated chick embryos (Figs. 3.4.2 and 3.4.5). The fully differentiated osteoblasts were in intimate contact with each other in bones of both the groups. The osteoblasts showed a normal complement of organelles in the controls as well as B-APN treated chick embryos (Figs. 3.5.2 and 3.4.5). The cells were cuboidal in shape and showed round nuclei (Figs. 3.5.1 and 3.5.4). The osteoblasts also showed an extensive network of rough endoplasmic reticulum studded with ribosomes (Figs. 3.5.3, 6). The Golgi apparatus was also quite extensive (Figs. 3.5.3 and 3.5.1).

The cells which were not immediately next to the surface of osteoid were elongated and showed very little endoplasmic reticulum (Figs. 3.5.1, 4). The cells which were close to bone surface showed many mitochondria per cell while cells away from the forming surface showed only few mitochondria (Figs. 3.5.1, 6).

The dark lysosome like bodies were observed in the controls as well as B-APN treated embryonic bones (Figs. 3.5.3, 5).

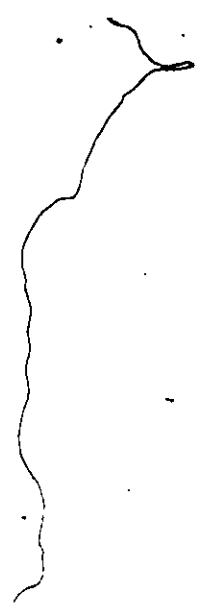
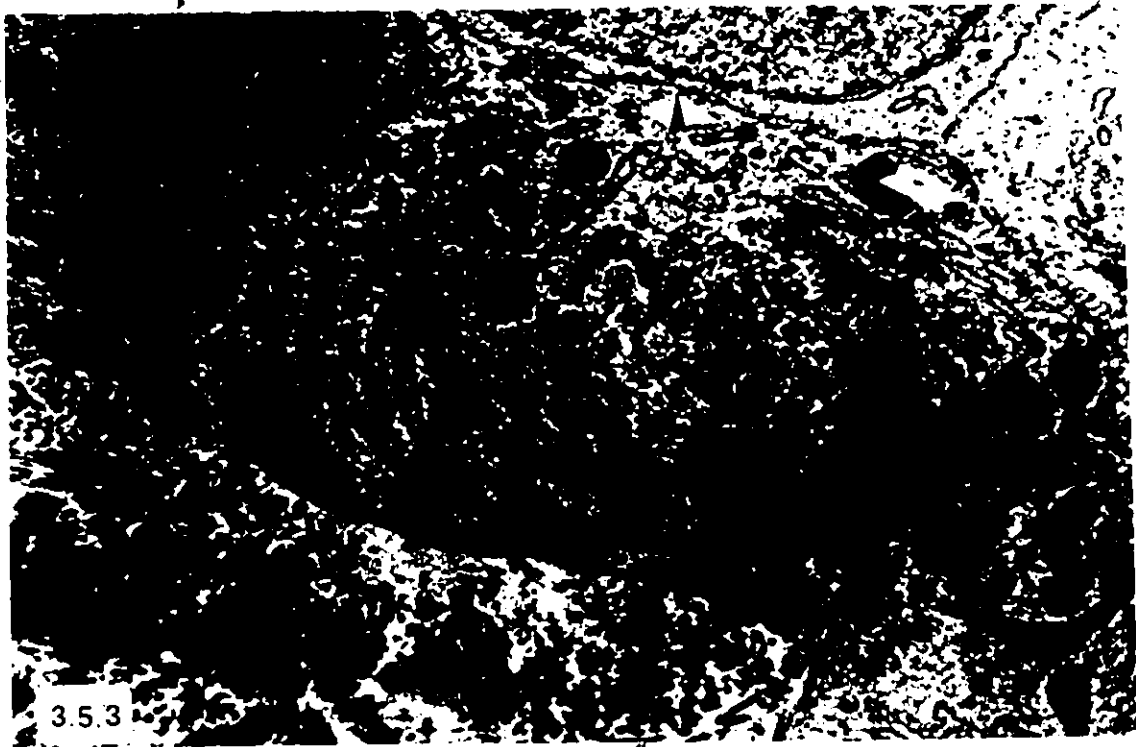
The osteoblasts forming the bone membrane were lying adjacent to each other and showed free running spaces between their surfaces in both the groups (Figs. 3.5.3, 4). These spaces have been described as "open canals" (Talmage, 1970). There were no discernible differences in width of these open canals between the controls and B-APN treated chick embryo bones (Figs. 3.5.4, 6). Frequently, the adjacent cell spacings were interrupted by small desmosomes joining the cells (Figs. 3.5.8).

There was always a zone of preosseous tissue between the bone itself and the cells of the bone membrane in the controls as well as B-APN treated embryonic bones (Figs. 3.5.1, 4). The preosseous tissue consisted of collagen fibrils, which were seen in cross and longitudinal sections with their characteristic banding pattern in both the groups (Fig. 3.5.2, 6). These fibrils were arranged haphazardly embedded in the ground substance (Figs. 3.5.3, 4). The matrix vesicles were also observed in both the groups (Figs. 3.5.3, 6).



35.1

Fig. 3.5.1. Bone membrane. An electronmicrograph from a part of the cross section of the control chick embryonic tibia. Mineralization front is marked (mf) and pre osseous zone is indicated by asterisk. The plasma membranes of osteoblasts (OB) are in apposition with each other (arrow heads). The osteoblasts show an extensive network of rough surface endoplasmic reticulum and Golgi apparatus (g). At the left side of the electronmicrograph are fibroblasts (Fb) which are devoid of a well developed endoplasmic reticulum and Golgi complexes X 10,690.



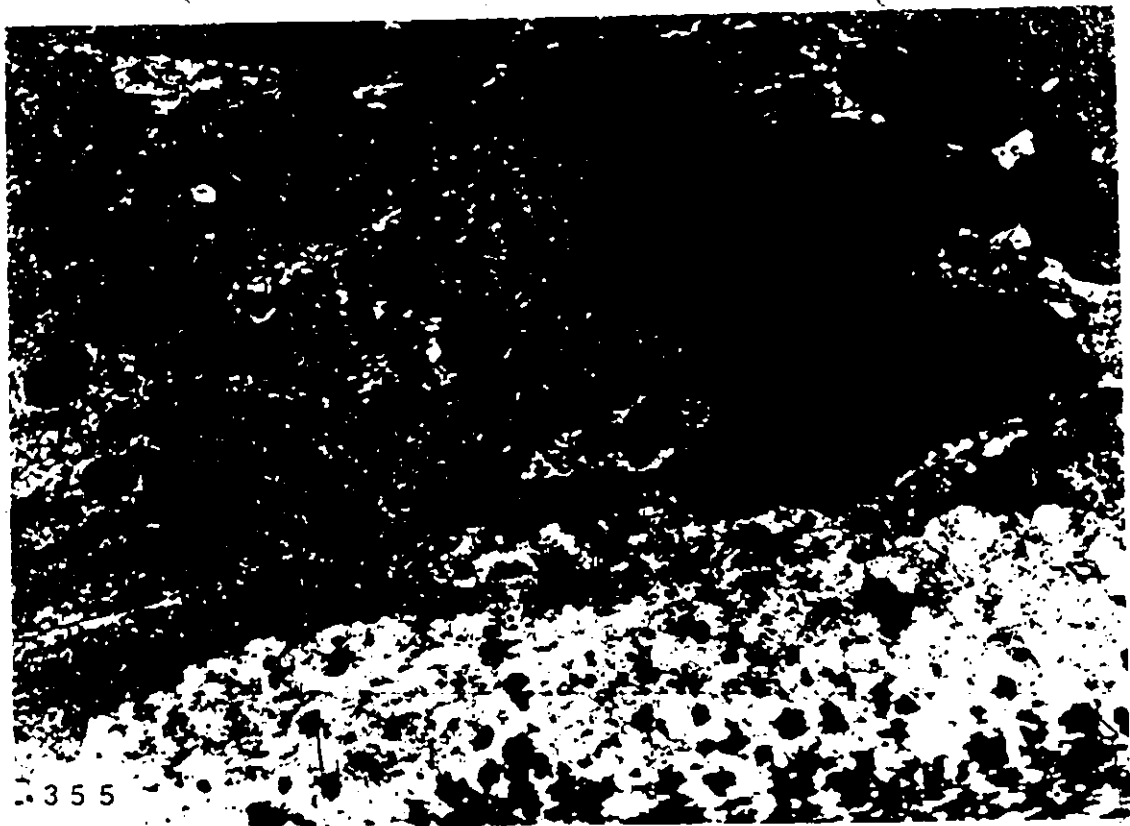
Both electronmicrographs from the controls.

Fig. 3.5.2. An area similar to Fig. 3.5.1. The pre osseous zone (asterisk) shows well defined banding pattern of collagen fibrils and osteoblasts (OB) forming an anatomical arrangement of the bone membrane. The plasma membranes of adjacent osteoblasts are closely apposed to each other (arrow heads). The osteoblasts show a normal components of intracellular organelles including mitochondria. The space between the plasma membranes of two osteoblasts (known as open canals) is indicated by an arrow X 16,100.

Fig. 3.5.3. An area similar to Fig. 3.5.1. The pre osseous zone (asterisk) shows collagen fibrils cut in longitudinal and cross sections and the matrix vesicles (arrow). The osteoblasts forming bone membrane show rough surface endoplasmic reticulum (er), Golgi (g) and many mitochondria. The plasma membranes of osteoblasts are in close apposition to each other (arrow heads) X 16,100.



3.5.4



3.5.5

Fig. 3.5.4. Bone membrane. The osteoblasts (OB) forming an anatomical arrangement known as the bone membrane in B-APN treated chick embryonic tibia. The cells are lining pre osseous zone (asterisk) and show a full complement of organelles including mitochondria (m). The plasma membranes of adjacent osteoblasts are in apposition with each other (arrow heads) and the communication with open canal is indicated by an arrow X 11,400.

Fig. 3.5.5 An area similar to Fig. 3.5.4 from B-APN treated embryos. Pre osseous zone (asterisk) shows collagen fibrils cut in cross and longitudinal sections. The plasma membranes of the adjacent osteoblasts (OB) are in apposition with each other (arrow heads) X 16,100.

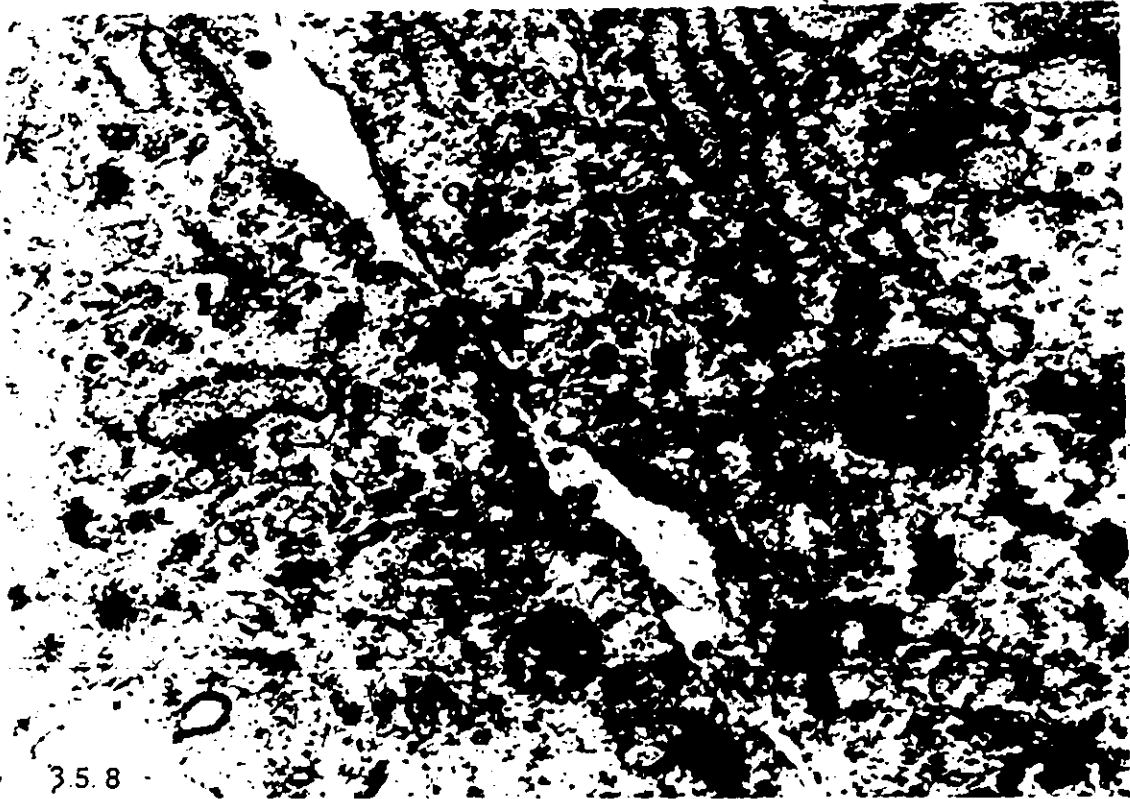


3.5.6

Fig. 3:5.6. A higher power electronmicrograph of an area similar to Figs. 3.5.4 and 3.5.5. In pre osseous zone (asterisk) are seen collagen fibrils cut in longitudinal and cross sections and matrix vesicles (arrow). The osteoblasts (OB) show a normal complement of organelles and the plasma membranes of adjacent cells are in apposition with each other (arrow heads) X 27,360. B-APN treated material.



3.5.7



3.5.8

Both electronmicrographs are from the controls.

Fig. 3.5.7. A high power electronmicrograph of three adjacent osteoblasts (OB) forming the bone membrane. The plasma membranes of cells are lying close to each other (arrow heads) and the space between them open canals, is indicated by an arrow X 51,300.

Fig. 3.5.8. An electronmicrograph of an area similar to Fig. 3.5.7. The osteoblasts (OB) are lying close to each other (arrow heads) and at certain places their plasma membranes are joined by a gap junction (big arrows) X 51,300.

3.6 EFFECTS OF B-AMINOPROPIONITRILE ON STRUCTURES IN THE ZONE OF THE INITIAL MINERALIZATION

3.6.1 Zone of Initial Mineralization

The initial phases of mineralization have been studied extensively in recent years with the help of TEM and SEM (Gay and Schraer, 1975; Gay, 1977; Sela et al., 1978; Sela and Bab, 1979).

The process involves the budding of matrix vesicles from osteoblasts or chondroblasts and concentration of calcium and phosphate in these vesicles and formation of apatite crystals. The apatite crystals grow and the membrane of vesicles rupture and the apatite crystals radiate outside and that probably leads to deposition of more minerals (Anderson, 1973). These events take place even in calcification of many other tissues under pathological conditions (Sarkar and Uhtoff, 1978, Sela and Bab, 1979, Freidman et al., 1980, Mann et al., 1980).

In our study of initial phases of the bone mineralization in chick embryo we observed similar structures described by earlier investigators.

3.6.2 Control Material

The osteoblasts were lying close to the preosseous zone. These cells showed all the signs of a metabolically active cell in the controls. The rough surface endoplasmic reticulum was very extensive and studded with ribosomes. The Golgi complexes of the cells were indicative of equally intense activity of these

cells (Figs. 3.6.1, 3.6.2, 3.6.3). The mitochondria could be seen in the osteoblasts and they were full of mitochondrial cristae (Figs. 3.6.1, 3.6.2 and 3.6.3).

In the preosseous zone, the matrix vesicles could be seen in association with the plasma membranes of the control material (Figs. 3.6.4 and 3.6.5). The matrix vesicles were bounded by a trilaminar membrane. The matrix vesicles situated closer in association with the osteoblast plasma membranes showed no mineral deposits in the material from the controls (Figs. 3.6.5 and 3.6.6). However, the matrix vesicles situated in the osteoid of bone matrix showed small amounts of mineral deposits (Figs. 3.6.6 and 3.6.7). The matrix vesicles, closer to cells, showed the mineral deposits which were well contained in the enveloping membrane (Figs. 3.6.7, 3.6.8 and 3.6.9). In the matrix vesicles closer to mineralization the complete envelope of trilaminar membrane was not visible and the apatite crystals were seen to radiate outward from the matrix vesicles (Figs. 3.6.7 and 3.6.10). These structures actually resembled the bone nodules described by Bernard and Pease, (1969) and Bonucci, (1970).

The collagen fibrils, in the preosseous zone, were not arranged in discrete layers whose fibrils would be oriented in a particular direction but were arranged in an irregular fashion and thus could be seen cut in cross as well as longitudinal sections in this material (Figs. 3.6.1, 3.6.4, 3.6.6 and 3.6.7).

These fibrils showed their characteristic banding pattern (Figs. 3.6.7, 3.6.8, 3.6.9 and 3.6.10). The presence of needle like apatite crystals on collagen fibrils has been shown by previous authors. A relationship of apatite crystals and banding pattern of the collagen fibrils has also been mentioned (Fitton-Jackson, 1957; Glimcher and Krane, 1968). In this material we did not observe any apatite crystal deposits on the collagen fibrils in the osteoid (Figs. 3.6.6 and 3.6.10). The collagen fibrils in the fully mineralized matrix were covered with mineral deposits and this was true even at the junction of osteoid with the mineralized matrix. Such deposits in association with collagen fibrils were always seen towards the mineralized matrix rather than towards the osteoblasts (Figs. 3.6.4 and 3.6.7).

In the preosseous zone a flocculent material was also observed in this material (Figs. 3.6.5, 3.6.6 and 3.6.7). However, no mineral was present in relation to this flocculent material (Figs. 3.6.5, 3.6.6 and 3.6.7).

3.6.3 Bones From B-APN Treated Embryos

The ultrastructural evaluation of the B-APN treated embryonic bones showed structures similar to the controls. The osteoblasts were lying close to preosseous zone. These cells showed all signs of a metabolically active cells in the B-APN treated material and there were no differences discernible as compared to the controls. The rough surface endoplasmic reticulum and Golgi apparatus were comparable to the controls (Figs. 3.6.11,

3.6.12 and 3.6.15). The cells contained many mitochondria and these mitochondria were full of cristae and did not show any signs of toxicity of B-APN (Figs. 3.6.11 and 3.6.12). The only discernible difference in the morphology of the osteoblast was that osteoblasts lying close to preosseous zone showed more lysosome like bodies in B-APN treated material (Fig. 3.6.11) although these bodies were present in the osteoblasts of the controls as well.

In the preosseous zone, the matrix vesicles could be seen in association with the plasma membranes of the osteoblasts and were similar to the structures observed in the controls (Figs. 3.6.13 and 3.6.14). The matrix vesicles were bounded by a trilaminar membrane. The matrix vesicles situated closer in association with the osteoblasts membrane showed no mineral deposits in B-APN treated material (Figs. 3.6.13 and 3.6.14) and these were similar to the ones observed in the controls. However the matrix vesicles situated in the osteoid of the bone matrix showed small amounts of mineral deposit (Figs. 3.6.13 and 3.6.14). The matrix vesicles, closer to cells, showed mineral deposits which were well contained in the enveloping membrane (Figs. 3.6.17 and 3.6.18). In the matrix vesicles closer to mineralization the complete envelope of trilaminar membrane was not visible and the apatite crystals were seen to radiate outward from the matrix vesicles (Figs. 3.6.17 and 3.6.19). These structures were similar to the bone nodules observed in the controls.

The collagen fibrils, in the preosseous zone were seen cut in cross as well as longitudinal sections (Figs. 3.6.17, 3.6.18 and 3.6.19). Their arrangement and appearance were similar to the ones observed in the controls. The banding pattern was also comparable to the controls (Figs. 3.6.13, 3.6.17 and 3.6.20). As seen in the controls no relationship was found between the collagen fibrils and apatite crystals in the B-APN treated material either (Figs. 3.6.13, 3.6.17, 3.6.18, 3.6.19 and 3.6.20). Whenever the mineral deposits were present on the collagen fibrils, it was only in the region of fully mineralized bone matrix or at the junction of the osteoid and the mineralizing front (Figs. 3.6.17 and 3.6.19).

In the preosseous zone a flocculent material, similar to the controls, was also observed (Figs. 3.6.17, 3.6.18 and 3.6.19). However, as described in the controls, no relationship between the mineral deposits and flocculent material was evident either.



3.6.1



Fig. 3.6.1. A section through an area of the initial mineralization from control chick embryonic tibia. In pre osseous zone (asterisk), seen are the collagen fibrils cut in cross and longitudinal sections with their characteristic banding pattern and matrix vesicles (small triangles). The osteoblasts (OB) show extensive network of rough endoplasmic reticulum (er), Golgi complexes (arrow heads) and mitochondria (m). The spaces between adjacent osteoblasts (open canal) is indicated by an arrow X 16, 100.



Fig. 3.6.2. A section similar to Fig. 3.6.1. Pre osseous zone is showing signs of mineralization. Mineralization front (mf) is moving towards osteoblasts which show normal complement of organelles, rough surface endoplasmic reticulum (er), Golgi (g) and mitochondria (m). X 16,100. Control material.



3.6.3

Fig. 3.6.3. An area similar to Fig. 3.6.2. In pre osseous zone the mineral deposits are seen. Some mineral has been lost during processing for ultra microtomy. Collagen fibrils are seen in cross as well as longitudinal sections. Also seen in the pre osseous zone are the matrix vesicles (arrow heads) X 16,100. Control material.



Fig. 3.6.4. A section through an area of the initial mineralization. Osteoblasts (OB) are seen to line the pre osseous zone (Pre Os.). Mineralizing front (mf) is seen to move towards cells. In pre osseous zone the collagen fibrils (cf) are seen in cross as well as longitudinal sections and their banding pattern is quite evident. From the surface of osteoblasts, the matrix vesicles are seen to take origin and matrix vesicles are also seen in pre osseous zone (arrow heads) X 16,100. Control material.



3.6.5

Fig. 3.6.5. Is from an area close to osteoblasts (OB) seen in Fig. 3.6.4. Matrix vesicles are seen budding from the osteoblast plasma membranes as well as in pre osseous zone (arrow heads). Note that matrix vesicles don't have any mineral deposits as yet. Collagen fibrils (Cf) are also seen in the pre osseous zone. Also seen in the pre osseous zone is a flocculent material (arrows) X 51,300. Control material.



Fig. 3.6.6. An area of the initial mineralization. Section is through the pre osseous zone seen in Fig. 3.6.4. Matrix vesicles (arrow heads) are seen in this zone. Note that matrix vesicles show accumulation of mineral deposits. Close to mineralization front the matrix vesicles have lost their trilaminar membrane and apatite crystals are radiating in the extracellular matrix. These structures are termed Bone Nodules (BN). Collagen fibrils are seen in cross as well as longitudinal sections. A flocculent material (arrows) is also seen in the pre osseous zone X 51,300. Control material.



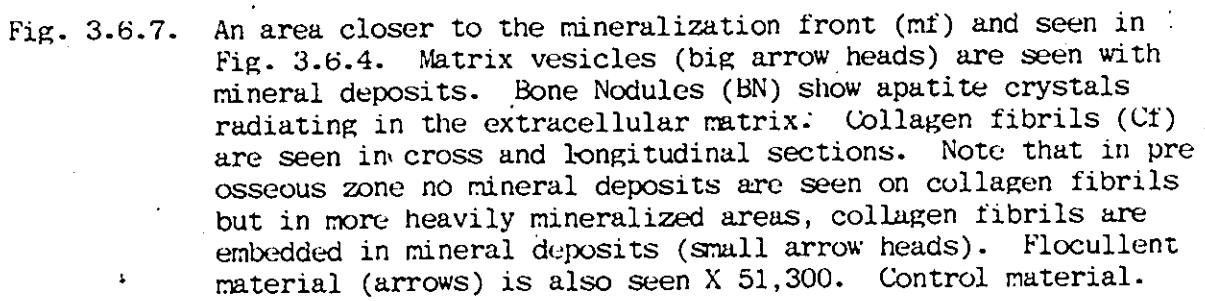


Fig. 3.6.7. An area closer to the mineralization front (mf) and seen in Fig. 3.6.4. Matrix vesicles (big arrow heads) are seen with mineral deposits. Bone Nodules (BN) show apatite crystals radiating in the extracellular matrix. Collagen fibrils (Cf) are seen in cross and longitudinal sections. Note that in preosseous zone no mineral deposits are seen on collagen fibrils but in more heavily mineralized areas, collagen fibrils are embedded in mineral deposits (small arrow heads). Flocculent material (arrows) is also seen X 51,300. Control material.

Handwritten mark or signature in the top left corner.



3.6.8

Fig. 3.678. An electronmicrograph of pre osseous zone. Note the needle like crystals in matrix vesicles (arrows). Also note that the collagen fibrils, seen in cross sections are free from any apatite crystal deposits X 51,300. Control material.



3.6.9


A micrograph showing a section of biological tissue, likely bone matrix. The image is mostly blank with some faint, scattered dark spots and a few small, dark, needle-like structures. There are some faint, irregular lines and shapes that might represent collagen fibrils or matrix vesicles, but they are not clearly defined. The overall appearance is that of a control material or a very low-magnification view of the tissue.

Fig. 3.6.9. A section similar to Fig. 3.6.8. Part of an osteoblast (OB) is seen. Matrix vesicles (pointed arrows) and collagen fibrils cut in cross and longitudinal sections are also seen. Note the needle like apatite crystals in matrix vesicles and absence of these crystals from the collagen fibrils. The crystals deposits in big bundles (big arrows) even seem to be away from collagen fibrils X 51,300. Control material.



3.6.10




Fig. 3.6.10. A section similar to Figs. 3.6.8 and 3.6.9. Matrix vesicles (arrows) show mineral deposits as apatite crystal while collagen fibrils (arrow heads) cut in cross and longitudinal sections are free from any crystal deposits X 51,300. Control material.



3611

Fig. 3.6.11. A section through pre osseous zone (asterisk) and osteoblasts lying adjacent to zone of initial mineralization, from the B-APN treated chick embryonic tibia. Osteoblasts seem to be very active in matrix synthesis and show extensive endoplasmic reticulum (er) and mitochondria (m) which are full of cristae. Dark lysosome like bodies (arrows) are also seen. In pre osseous zone (asterisk) collagen fibrils with their characteristic banding pattern are seen cut in longitudinal as well as cross sections X 16,100.

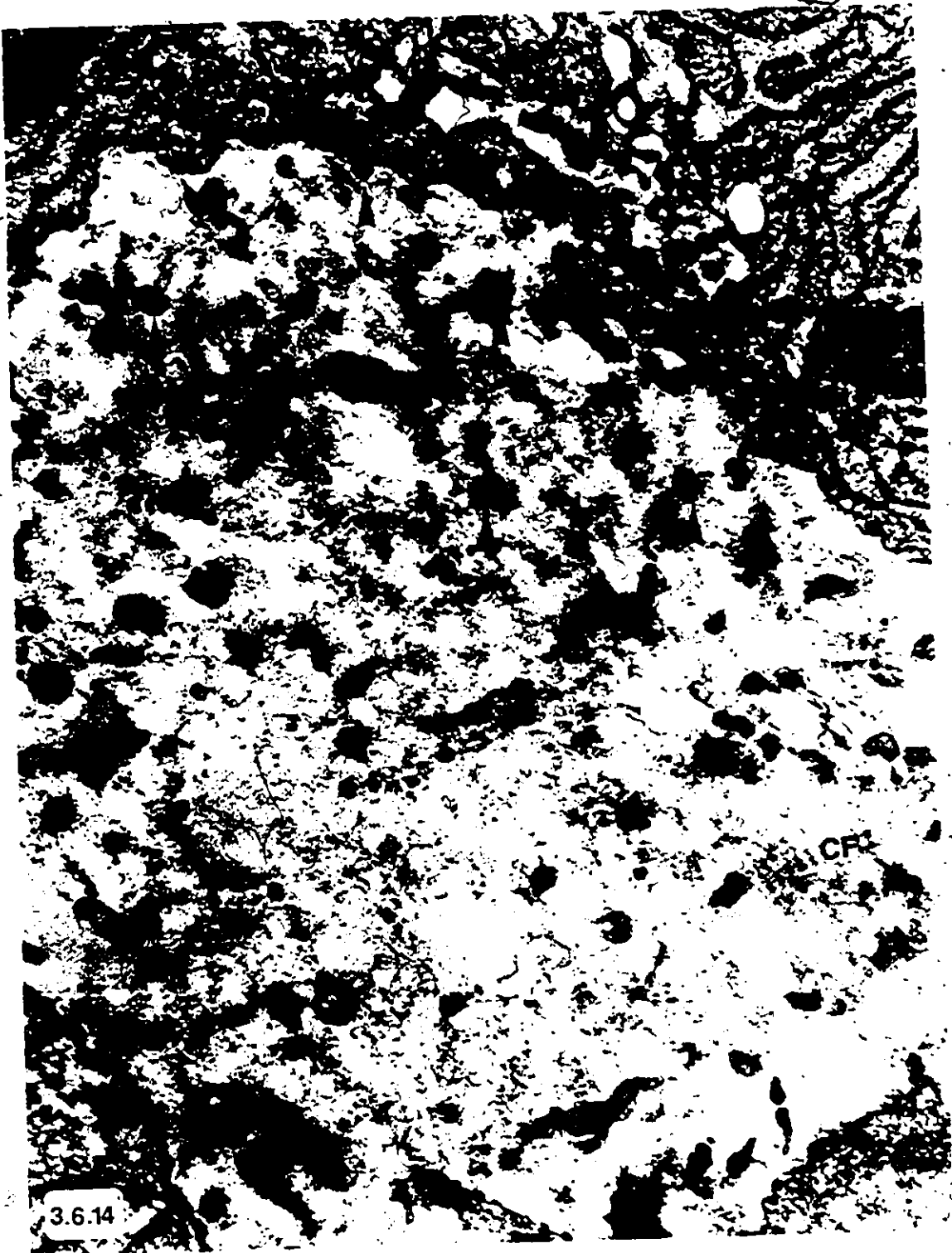


Fig. 3.6.12. A high power electronmicrograph similar to Fig. 3.6.11. Part of an osteoblasts is seen lying adjacent to pre osseous zone. Osteoblasts show well developed rough surface endoplasmic reticulum (er), mitochondria full of cristae (mit.) and a well developed Golgi apparatus (golgi). In pre osseous zone collagen fibrils are seen cut in longitudinal as well as cross sections. Matrix vesicles (arrow heads) with apatite crystals in side are also seen. Structures called bone nodules are indicated by BN and arrow X 51,300. B-APN treated material.



3.6.13

Fig. 3.6.13. A section similar to figure 3.6.12. Osteoblasts (OB) are lying adjacent to pre osseous zone. Pre osseous zone shows collagen fibrils (Cf) cut in mostly longitudinal sections. Matrix vesicles (arrow heads) with apatite crystals are seen in pre osseous zone. Note the absence of any apatite crystals on collagen fibrils. A flocculent material is also seen in the extracellular matrix X 51,300. B-APN treated material.



3.6.14

CFR

Fig. 3.6.14. A section showing part of two osteoblasts and the pre osseous zone. Note the budding of matrix vesicles from the plasma membrane of osteoblasts (arrow heads) and also in extracellular matrix (arrow heads). Collagen fibrils (Cf) are seen cut in cross as well as longitudinal sections. Note the absence of apatite crystals on the surface of collagen fibrils X 51,300. B-APN treated material.



36.15

Fig. 3.6.15. A section through the zone of initial mineralization and osteoblasts lying adjacent to pre osseous zone from B-APN treated chick embryonic tibia. Osteoblasts (OB) at the lower right corner of electronmicrograph is being surrounded by mineralizing matrix where ultimately it will become an osteocyte. Mineralizing front (Mf) is progressing towards the next layer of cells. Osteoblasts are full with rough endoplasmic reticulum, normal mitochondria and very extensive Golgi complex (arrow heads). In pre osseous zone the matrix vesicles (big arrows) and collagen fibrils (small arrows) are seen X 16,100.



3616

Fig. 3.6.16. A high power electronmicrograph of the area marked on Fig. 3.6.15. In the zone of initial mineralization matrix vesicles (arrows) can be seen budding from osteoblast (OB) at the lower half of picture and also in the pre osseous zone. Some of the matrix vesicles have already lost their enveloping membrane and become bone nodules (arrow heads). Collagen fibrils are also seen (Cf). Note the absence of apatite crystals in relation to collagen fibrils X 51,300.



Fig. 3.6.17. A high power electronmicrograph of a section through mineralizing front (Mf), pre osseous zone and the osteoblasts (OB) from a B-APN treated chick embryonic tibia. Matrix vesicles (arrow heads) are seen in association with plasma membranes of osteoblasts. Matrix vesicles (seen at the upper left side of picture) do not show any mineral deposits. At the middle of the picture a protrudation of osteoblasts is seen which will probably pinch off and become a matrix vesicle X 51,300.



3 6 18

Fig. 3.6.18. A section through pre osseous zone (asterisk) and an osteoblast lying adjacent to it. A lysosome like body containing granular material is seen (arrow) in osteoblast. Pre osseous zone shows matrix vesicles (arrow heads) and collagen fibrils. Matrix vesicles which are closer to cell surface show very little mineral deposits X 51,300. B-APN treated material.



MF

36.19

Fig. 3.6.19. An electronmicrograph of a section through mineralization front (Mf), pre osseous zone and part of an osteoblast (OB) lying adjacent to pre osseous zone from B-APN treated material. Matrix vesicles (arrow heads) and collagen fibrils are seen in pre osseous zone. Matrix vesicles show mineral deposits. Collagen fibrils only close to mineralization front are covered by needle like apatite crystals X 51,300.



3.6.20

Fig. 3.6.20. A section passing through the pre osseous zone of B-APN treated chick embryonic tibia. Part of the osteoblasts (OB) are seen adjacent to osteoid. In pre osseous zone are seen collagen fibrils (Cf) and the matrix vesicles (arrow heads). Mineralization front (Mf) shows some loss of mineral due to preparation procedure. Note that mineral deposits are not present in association with collagen fibrils X 82,080.

CHAPTER 4.

DISCUSSION

4.1 PERIODONTAL LIGAMENT (PDL) AND B-APN

4.1.1 Light Microscopy

In these studies the most striking changes observed with light microscopy, in periodontal ligament with B-APN treatment, were a palisade arrangement of cells and the hyalinization of the extracellular matrix. Similar changes had been described by earlier investigators (Sciaky and Ungar, 1961; Krikos et al., 1965; Barrington and Meyer, 1966; Shoshan et al., 1969; and Yoshikawa, 1971) although extent of these alterations in different regions of the periodontal ligament had not been mentioned. In this investigation a special effort was made to see the effects of B-APN treatment on various regions of the periodontal ligament. Although the fibres in different regions of the periodontal ligament perform specific functions in relation to the tooth movement the horizontal and oblique sets of fibres have been mentioned as the main bearers of the masticatory stress (Provenza, 1972). Our investigations have shown that it was the region of horizontal and oblique fibres where the severest changes were observed. These findings correlate with the earlier findings of Krikos et al., (1965) that removal of an antagonist of a tooth will eliminate these alterations and the periodontal ligament will be normal again.

The insertion of collagen fibre bundles into the alveolar bone and dentin or cementum is also a major factor for optimal functional ability of the periodontal ligament (Glickman, 1972).

The present investigation showed an irregular arrangement of the fibre insertion into alveolar bone in B-APN treated rats as compared to the controls. The disruption or improper insertion of these fibres could cause an impaired functional capability of the periodontal ligament (Glickman, 1972). Furthermore, the present investigation showed that the effects of B-APN on the alveolar crest fibres and the apical fibres are not as severe as on the horizontal and oblique fibres. This points toward a differential effect of B-APN on different regions of periodontal ligament according to their function.

4.1.2 Electronmicroscopy

The ultrastructural study again showed aggregation of cells into groups. However, the most explicit changes were those seen in the mitochondria of the fibroblasts of the periodontal ligament. Similar changes in mitochondria of the periodontal ligament fibroblasts have been produced by increasing the stress with orthodontic treatment in rats (Rygh, 1972). Other physiological studies have shown an impairment of oxidative metabolism of cells by B-APN treatment (Juva et al., 1959, Clermons, 1966, Elders et al., 1973). The alteration of morphology of the chondrocyte mitochondria, in fetal rib cartilage, has been attributed to the direct toxic effects of B-APN (Wiley and Joneja, 1978).

In the present investigation the osteoblasts at the surface of alveolar bone, in periodontal ligament, are free from these effects on mitochondria. Secondly, the cells in different

regions show a differential extent of these changes in mitochondrial morphology and these changes of course are not artefacts, as the muscle tissue kept with the periodontal ligament is free from these alterations. The most severe alterations are seen only in the region of maximum masticatory forces, the region of horizontal and oblique fibres. The optimal functions of connective tissue requires high tensile strength developed in collagen fibrils (Tanzer, 1973). It is known that the collagen produced under the effect of B-APN is deficient in tensile strength. On the basis of the above discussion, it appears that the toxic effects of B-APN on oxidative metabolism become accentuated due to the masticatory forces which have become traumatic due to the loss of tensile strength.

Unaltered morphology of endoplasmic reticulum and Golgi apparatus of the fibroblasts in lathrytic periodontal ligament correlates with the findings of previous workers (Golub et al., 1968; Alco et al., 1974) that the B-APN does not cause any quantitative changes in the synthesis of collagen. This study also did not show any quantitative effects of B-APN on the various components of the extracellular matrix in periodontal ligament.

Our statistical evaluation of the effects of E-APN, on the diameter of collagen fibres, showed that the higher percentage of collagen fibrils have a significantly smaller diameter as compared to the controls.

These data are similar to earlier observation of Matukas et al., (1967) and Wiley and Joneja, (1978), although their studies were on collagen fibrils of the cartilage, that these fibrils formed under the effect of B-APN are of a smaller diameter. The increase in the diameter of collagen fibrils takes place by addition of tropocollagen molecules from ground substance of the extracellular matrix (Olson and Low, 1970). Inter and intra molecular cross linkages, inhibited by B-APN, play a role in the extracellular maturation of the collagen fibrils (Piez, 1968). The smaller diameter of collagen fibrils of the periodontal ligament, demonstrated in the present investigation, thus correlates with the fact that B-APN inhibits these molecular cross linkages of collagen.

4.1.3 Microradiography

The microradiographs prepared from whole one side of the rat mandible and the from the longitudinal sections of rat mandible showed, after four weeks of B-APN treatment, that the radiodensity of the alveolar bone was lower as compared to the controls. Interdental and inter-radicular septae were less radio-opaque in the B-APN treated rat mandibles as compared to the controls. The medullary spaces of alveolar bone have increased in size and seem to communicate with the periodontal ligament space. On the whole a reduced amount of bone tissue was present in the mandibles of rats treated with B-APN as compared to the controls.

The alveolar bone is least stable of the periodontal tissues and its structure is in a constant state of flux (Manson, 1964). The physiological status of alveolar bone is maintained by a sensitive balance between the bone formation and resorption (Carranza and Cabirini, 1967). The reduced amount of bone tissue thus results from an imbalance between the bone formation and resorption. This imbalance can be the result of any of the following three possibilities.

- (i) Increased resorption in the presence of normal or increased formation. The latter being still less than the increase in resorption.
- (ii) Decreased formation in the presence of normal resorption.
- (iii) Increased resorption combined with decreased formation.

Our studies, both morphometric and radioisotopic (Sandhu and Jande, 1982 b), to be discussed later, on the effects of B-APN on the tibial bone formation have shown that B-APN does not affect the amount of bone matrix formed (Sandhu and Jande, 1982 a). Earlier investigators have also shown, in an in vitro study, that B-APN does not affect the amount of bone matrix formed (Golub et al., 1968). In view of this earlier information and our own experiments in rats it seems reasonable to conclude that the lower amount of bone tissue present in rat mandibles, after B-APN treatment, is most probably due to the increased resorption of alveolar bone.

4.1.4 Histochemistry

In this investigation the histochemical evaluation of effects of the B-APN on alveolar bone and periodontal ligament showed that the acid phosphatase activity was higher in osteoclasts, osteoblasts, osteocytes and fibroblasts of the rats treated with B-APN as compared to the controls.

The enzyme acid phosphatase is a hydrolytic enzyme and is present in a variety of cells including osteoclasts, osteoblasts and fibroblasts. In histochemical studies it is used as a marker for other hydrolytic enzymes (Deans et al., 1972). The presence of acid phosphatase and other hydrolytic enzymes in the cells at the surfaces where resorption takes place, the abundant presence of these enzymes in the bone resorptive cells and the rise in the activity of acid phosphatase in response to certain hormonal factors which are known to increase resorption, has prompted many investigators to ascribe it a role mainly in the bone resorption (Vaes, 1965, 1966, 1968, Doty et al., 1968).

It is generally agreed that the osteoclasts, which have a large number of lysosomes, are very rich in hydrolytic enzymes as compared to other cells like osteoblasts and osteocytes (Holtrop, 1977). The presence of osteoclasts at the resorptive surfaces of the bone, in Howship's lacunae, is tantamount to bone resorption.

In the normal periodontium the physiological masticatory forces are a stimulant for bone formation and resorption (Glick-

man et al., 1970). However, if the occlusal forces exceed the adaptive capacity of the periodontium especially compounded with decreased tensile strength of collagen fibrils, tissue injury results (Adrien, 1933; Balfe et al., 1938; Coolidge, 1938; Glickman and Weiss, 1955). Thus the increased resorption could be due to such trauma. It has also been shown that physical pressure increases the resorption (Manson, 1964).

In the present investigations, the microradiographic as well as the histochemical findings point towards reduced amount of bone tissue in the B-APN treated rat mandibles due to increased bone resorption. It is known that B-APN reduces the tensile strength of collagen (Piez, 1968). It appears that tensile strength deficient collagen fibres cannot maintain the functional integrity of the periodontium and normal masticatory forces become traumatic. The derangement of certain fibrils of the PDL in the B-APN fed rats, supports this assumption.

The presence of activity of acid phosphatase and other hydrolytic enzymes, in osteoblasts, has been linked to the synthesis of various components of the bone matrix (De Duve, 1963; Garant and Prockop, 1972; Jande and Grosso, 1975). As it has been shown that with B-APN treatment there is no change in synthesis of the bone matrix (Golub et al., 1968; Sandhu and Jande, 1982) the activity of acid phosphatase, which appeared to have increased with B-APN treatment, in osteoblasts can only be due to toxic effects of B-APN. It is known that in response to certain

drugs or in certain pathological situations the activity of acid phosphatase becomes high (Susi et al., 1966).

Also in the present study, the fibroblasts of the periodontal ligament, those that are arranged in groups, show a greater acid phosphatase reaction as compared to the controls. In the periodontal ligament the presence of acid phosphatase has been shown in fibroblasts and it has been linked to "fibroclasis" (Garant, 1975). Here again these effects were maximum in the region of maximum masticatory forces. The increased activity of acid phosphatase and possibly other hydrolytic enzymes in fibroblasts of the region of maximum masticatory forces, with B-APN treatment, is either due to increased turnover of collagen and thus increased requirement of acid phosphatase and other hydrolytic enzymes for fibroclasis or is due to the direct toxic effects of B-APN as mentioned for the osteoblasts.

Periodontosis is defined as a periodontal disease caused by systemic factors in the presence or absence of local infection. The radiographic signs of this disease include loss of alveolar bone by resorption, increase in size of medullary spaces and wavy or potted appearance of inter radicular septum (Glickman, 1972). The disruption of collagen fibrils in periodontal ligament is another factor involved in periodontosis (Glickman, 1972). Collagen diseases have also been implicated in promoting the periodontal disease (Kerr, 1962). The role of dietetic factors has been ignored so far in promoting this periodontal

disease. With the B-APN treatment the rats showed, in their periodontium, similar changes as described above. On the basis of these observations we suggest that dietetic factors be considered a strong possibility in producing the conditions, which if unchecked, can lead to periodontal disease especially in under privileged populations or in situations of famine when sweet peas might become a major ingredient of the diet.

4.2 EFFECTS OF B-APN ON DYNAMICS OF ^3H -Proline and ^3H -Tetracycline AND VALIDITY OF THE INCORPORATION OF THESE ISOTOPES AS MEASURES OF BONE MATRIX FORMATION AND ITS MINERALIZATION

4.2.1 Validity of ^3H -Proline Incorporation as a Measure of Bone Matrix Formation

It is a quantitative method that evaluates the formation of bone matrix by assaying labelled hydroxy-proline in bone after administration of its precursor, the labelled proline, as used by Flanagan and Nicholas (1962). This method is based on the fact that hydroxyproline is formed by hydroxylation of peptide bound proline residues in α -chains of collagen molecule and hydroxyproline is exclusively found in the collagen of animal tissue (Udenfriend, 1966). Thus ^3H -proline injected to animals can later be recovered, in very high percentage, from collagen (Marks, 1969; Sodek et al., 1977). Numerous authors, using autoradiography and by measuring ^3H -proline radioactivity by liquid scintillation, at certain periods after injection, have shown that ^3H -proline incorporates itself into bone and can be considered an indicator of the bone matrix formation (Leblond, 1965; Walker, 1966; Frank and Frank, 1969; Jowsey, 1971; Turner et al., 1977). In an exclusive experiment it has been unequivocally shown that the incorporation of ^3H -proline into bone is a true indicator of the bone matrix formation (Marks, 1969).

4.2.2 ^3H -Proline Dynamics

The present study shows that, after injection of ^3H -proline in rats, the counts in the bone are initially very high. The CPM continue to increase up to 12 hours after administration but then start decreasing abruptly and stabilize at about 36 hours and do not change appreciably thereafter.

In order to get a clear picture of incorporation of ^3H -proline into bone, the sacrifice time of animals, after injections, is very critical. The time that will give a true indication of the amount of ^3H -proline incorporated will be when all the ^3H -proline which could incorporate has been incorporated into newly formed bone matrix but resorption of the bone in those areas has not started.

There have been numerous studies on bone matrix collagen formation using labelled proline and glycine (Carneiro and Leblond, 1959; Young and Greulich, 1963; Ross and Benditt, 1963; Leblond, 1963). In a review article Firschein, (1969) suggested that labelled proline incorporation is a more specific indicator of collagen formation in bone than labelled glycine. Leblond and Weinstock, (1974) have studied ^3H -proline radioactivity in blood and its incorporation, after injection, into bones with autoradiography. They found that initially, the radioactivity was higher in blood and the bone ^3H -Proline label was in osteoblasts. They further showed that as the time lapsed the radioactivity in blood decreased and along with it the ^3H -label

could be seen in newly forming osteoid of the bone matrix. On the basis of this autoradiographic study they concluded that ^3H -proline, after administration to animals, becomes a part of newly formed bone matrix in about 24-36 hours.

Firschein, (1967 a, b, 1969) studied the incorporation of ^3H -proline into various bones of the rats and found that the radioactivity becomes stable at about 1-2 days and does not change appreciably thereafter unless left over a period of weeks. He further observed that once incorporated into bone matrix, ^3H -proline radioactivity decreases only because of resorption.

Our studies clearly showed a stabilizing of the label by 36 hours after dosing and as discussed above this seems to be due to the fact that the labels by that time had become a part of the bone matrix. As the newly formed bone is on the surfaces of new trabeculae there does not appear to be any complication because of the resorption as the time interval is too short for resorption and also the resorption generally occurs on the surfaces of older trabeculae.

4.2.3 Validity of ^3H -Tetracycline As An Indicator of Initial Mineralization

In the last 20 years numerous investigators have studied bone dynamics by labelling it with the antibiotic tetracycline. They have suggested that the tetracycline incorporates into newly forming bone at the time of mineralization (Milch et al., 1958; Harris et al., 1962; Berquist, 1962; Berquist and Hulth, 1963). This incorporation of tetracycline is believed to be due to its chelating properties. It forms complexes with Ca at the surface of newly formed apatite crystals (Urist and Ibsen, 1963; Steendizk, 1964; Ibsen and Urist, 1964). The tetracycline has much higher affinity to bind with Ca on the surface of newly formed crystals as compared to the old crystals.

Further it has been found that tetracycline does not incorporate into organic bone matrix to any appreciable amount and on treatment with EDTA, which removes only the mineral part, fluorescence of tetracycline from the mineralized bone is also lost (Urist and Ibsen, 1963; Steendizk, 1964). Many authors have combined ^{45}Ca autoradiography with the cold tetracycline labelling and have found that the site of incorporation of both labels is the newly formed bone undergoing mineralization (Rowland et al., 1962. Urist and Ibsen, 1964).

Klein and his collaborators (Klein and Jackman, 1972, 1976) have used ^3H -Tetracycline in their study on bone resorption. Their results show that tetracycline incorporates in bone mineral

and forms very strong complexes and it is not removed until re-sorption takes place. Similar findings have been reported on the basis of cold tetracycline incorporation (Frost, 1963). Lacroix, (1971) has suggested that fluorescent markers of bone, like tetracycline, are markers of sudden calcification. Baylink et al., (1970, 1972), elaborating further, have suggested that the tetracycline incorporates into bone only with initial mineralization.

Trecharne and Brighton, (1979), on the basis of previous data and also taking into consideration their own experiments, have concluded that the tetracycline incorporation into bone is a true indication of mineralization of newly formed bone matrix. It is very important to point out that the use of ^3H -tetracycline has an advantage over that of ^{45}Ca as a marker of the mineralization. The former does not diffuse into and deposit on crystal surfaces of previous mineralization but it gets absorbed on surfaces of the newly forming crystals. ^{45}Ca on the other hand exchanges with the calcium of bone at sites other than the new mineralization (Klein and Jackman, 1972).

It thus seems reasonable to conclude that the tetracycline incorporates into newly forming bone and its incorporation can be taken as an indication of the mineralization of newly formed bone matrix.

4.2.4 ^3H -Tetracycline Dynamics

The present study on ^3H -Tetracycline dynamics showed that, after injection of labelled tetracycline to rats, the radio-

activity in bone soon after dosing was quite high. The CPM/100 mg wet tibial weight continued to increase up to 12 hours after administration but then started to decrease and stabilize at about 36 hours and did not change appreciably thereafter.

The author is not aware of any other study using ^3H -tetracycline in investigation of mineral dynamics of bone except that on bone resorption done by Klein and his associates (Klein and Jackman, 1972; 1976), although there have been many reports on mineral deposition in bones using ^{45}Ca , ^{47}Ca and ^{85}Sr (Uitto and Laitinen, 1966; Firschein, 1969; Lacroix, 1971). All these studies show that the radioactivity in the bone due to these isotopes was high initially but decreased with time. Most of these studies have been done over a very short period of time (1-6 hours). In the shorter time span it appears that the initial high radioactivity is due to presence of the isotopes in blood and interstitial fluids of the bone and is still in a labile form. Our results are consistent with the views of earlier authors (Uitto and Laitinen, 1966; Lacroix, 1971), that the radioactivity in bones is higher initially but decreases with time.

Earlier workers although using different labels such as ^{45}Ca (Firschein, 1969) have shown that radioactivity becomes stable at about 1-2 days and does not change unless the resorption takes place. In experiments of longer duration the resorption of labelled bone or even unlabelled bone matrix will

undoubtedly complicate the experimental design. Thus the present study is consistent with the results of earlier investigators and it appears that at 36 hours, the sacrifice time of animals in later experiments, the ^3H -Tetracycline had become a part of newly mineralized matrix and also was not in a labile form.

4.2.5 Effects of B-APN on ^3H -Proline and ^3H -Tetracycline

Dynamics

The present study on ^3H -proline and ^3H -tetracycline dynamics in rats, after B-APN treatment for 3 weeks, showed that the pattern of radioactivity of both the isotopes in bone, does not change. (Figs. 3.2.1, 2, 3, 4).

In our knowledge these parameters have not been previously compared after B-APN treatment. Our findings that both the isotopes, ^3H -proline and ^3H -tetracycline, become a part of newly formed mineralizing osteoid in about similar periods in the normal and B-APN treated rats show that the B-APN treatment does not inhibit or expedite, in any way, the excretion or retention of these isotopes. This finding further eliminates the possibility that B-APN might have influenced the stabilization of these isotopes, in bone, and thus had effected the results of our experiments.

4.3 EFFECTS OF B-APN ON BONE MATRIX FORMATION AND ITS MINERALIZATION IN RATS

4.3.1 Effects on General Body Growth and The Growth of Bone at Organ Level

In this study, it is quite apparent from the comparison of total body weights and tibial lengths at 5, 10 and 20 days after B-APN treatment that this drug, at the doses used in the present investigation, does not effect the general body growth of the rats including that of the bones at organ level. Rosenquist et al., (1977) have reported similar findings although a higher dose of B-APN was used in the present investigations.

4.3.2 Effects on Linear Bone Apposition

The findings of cold tetracycline fluorescent labelling measurements of the amount of bone matrix formed, as shown by the amount of bone between the two consecutive labels, showed that the linear bone apposition at the periosteal surface of rat tibia was comparable in both the groups at all intervals during the present experiments. The cold tetracycline has been used to measure various parameters related to bone formation by numerous authors (Milch et al., 1958; Frost and Villaneuva, 1960; Berquist and Hulth, 1963; Baylink et al., 1970). The bands of tetracycline provide a mapping of events taking place in a specific period of time. Rosenquist et al., (1977) used the cold tetracycline to evaluate the effects of B-APN on the bone matrix formation and its mineralization and found that the rate of

initial mineralization was inhibited while all other parameters were normal.

The findings of the present study are consistent with those of Rosenquist et al., (1977) that the amount of bone matrix formed is not affected by B-APN, however, Rosenquist et al., (1977) did not publish the photomicrographs of sections from which they made their observations.

The fluorescent study further showed, in the controls as well as in B-APN treated rats, that bone matrix synthesis, as shown by the width of bone present between two labels, varies with the age of the animal. These findings are similar to a previously detailed study done on rates of bone matrix synthesis in different species by Simmons (1976).

4.3.3 Effects on ^3H -Proline Incorporation

An analysis of ^3H -proline incorporation, in the present investigation at different intervals, into tibias of the controls and B-APN treated rats showed that bones of both groups incorporated similar amounts of this isotope. As ^3H -proline incorporation is a true measure of bone matrix formation (discussed earlier) the results of this investigation show that, B-APN at the doses used, did not inhibit the synthesis of bone matrix. The fluorescent microscopic results (as discussed above) also support these data. It has also been shown earlier, in an in vitro study (Golub et al., 1968) that B-APN does not effect the bone matrix synthesis. Rosenquist et al., (1977)

provided a similar conclusion based upon the morphological measurements of tetracycline labelling. However, in the present investigation, a higher dose of B-APN was used as compared to the one used by Rosenquist et al., (1977). Furthermore the technique used in the present investigation is of higher sensitivity and accuracy and the findings of this highly quantitative technique correlate with those of morphological measurements of cold tetracycline labelling. Thus on the basis of previous findings of other investigators and on our own results it is quite well documented that B-APN, at the doses used, does not affect the quantity of the bone matrix formed.

4.3.4 Effects on ^3H -Tetracycline Incorporation

The present study showed, on analysis of ^3H -Tetracycline incorporation into the bones of the controls and B-APN treated rats at different intervals, that the incorporation of this isotope is significantly lower in bones of the rats treated with B-APN as compared to the controls at all intervals. It has already been established that the lathyrogen B-APN does not affect the dynamics of the tetracycline which could in turn affect its incorporation into bones. As discussed in an earlier section, it is quite well established that the incorporation of ^3H -tetracycline is a sensitive and accurate measure of the initial mineralization. The lower incorporation of ^3H -tetracycline into bones of rats treated with B-APN can thus be

attributed either to a smaller area of the matrix undergoing mineralization or to a lower amount of mineral per unit area of matrix synthesized. The amount of bone matrix, as indicated by ^3H -proline incorporation and measurements of cold tetracycline labelling, is almost similar in the controls and B-APN treated rat bones. The lower incorporation of ^3H -tetracycline thus seems only to be due to a lower degree of mineralization per unit area.

4.3.5 Variation in Synthesis of Bone Matrix With Age

In the present study, the results of scintillation counting experiment demonstrate in the controls as well as in B-APN treated rats, that the rates of bone matrix synthesis differs with the age of animals. These findings are similar to our findings of fluorescent study as mentioned above. In this study a larger incorporation of ^3H -Proline at day 20 of the experiment as compared to day 10 was observed in the controls as well as in B-APN treated rats. It is known that at about seven weeks the rats show a pubertal growth spurt and there is an elevation of plasma androgens levels (Kinson and Chung-Ching, 1971). The anabolic effects of the androgens are well known. These hormones increase the general rate of protein synthesis. So in view of this information it does not seem unreasonable to see a larger incorporation of ^3H -proline at day 20 as compared to day 10.

4.3.6 Comparison of Bone Matrix Synthesis and Its Mineralization

A comparison of bone matrix synthesis as shown by ^3H -proline incorporation and discussed earlier, and mineralization, indicated by ^3H -tetracycline incorporation in controls (i.e. ratio of ^3H -proline incorporated/ ^3H -tetracycline incorporation - 2.02/1.193) and B-APN treated rats (1.2/1.09) at day 5 clearly suggest an inhibition of about 35% in the mineralization (controls 1; B-APN 0.65). This inhibition was 31% at day 10 and decreased further to 9% by day 20. These data again suggest a lower extent of mineralization of the bone matrix formed under the effect of B-APN.

In summary the B-APN treatment, at the doses used, does not have any effect on synthesis of bone matrix but it does inhibit the initial bone mineralization. We have established a sensitive system, different from morphological techniques, by which the various parameters of the bone metabolism can be investigated with greater precision and accuracy. Also it can be shown by a very accurate and quantitative technique that B-APN inhibits the initial phases of mineralization and does not have any effect on the quantity of bone matrix formed and these findings correlate with morphological measurements.

4.4 EFFECTS OF B-APN ON CHICK BONE MATRIX FORMATION AND ITS

MINERALIZATION

4.4.1 Rationale Behind the Study on Chicks

A quantitative system to evaluate the effects of B-APN on various parameters of the bone metabolism having been established (Sandhu and Jande, 1982 a) as described in an earlier section, it was considered important to study the effect of B-APN on some other animals as well because species difference of sensitivity to B-APN had been suggested by Barrow et al., (1974). Also growth of the diaphyseal bone in chicks presents a simpler system than that of the rats because the bone growth mainly takes place on the periosteal surface and resorption is generally restricted to the endosteal surface (Jande, 1971). As the bone growth takes place only on the periosteal surface it was found easier to follow the tetracycline fluorescent bands. Also the innermost band, which represented the tetracycline dose at 0 day, if not resorbed until the end of experiment, can give information on the bone resorption due to B-APN treatment.

4.4.2 Difference in Sensitivity to B-APN

The present experiments on chicks show that there is a difference of sensitivity to B-APN based upon age and species. The four week old rats could tolerate a dose of 0.25% in their diet without any effects on the general body growth while in two week old chicks the dose had to be reduced to 1/5th of the dose given to rats. These findings are in agreement with those of

Barrow et al., (1974).

4.4.3 General Body Growth and the Growth of Bone at Organ Level

In the present study a comparison of total body weights and the tibial lengths at 7, 14 and 21 days after B-APN treatment showed that this drug, at the doses used in this experiment, does not affect the general body growth of the chicks and even that of the bones at the organ level.

4.4.4 Effects on Bone Ash Contents

The present study on ash contents of the chick bones showed that, after B-APN treatment, the bone ash content was lower at all intervals during the experiment as compared to the controls. These findings are in agreement with those of Rosenquist et al., (1977) and our own study on rats (Sandhu and Jande, 1982 a).

4.4.5 Effects on Bone Resorption

The microradiographic examination of tibial diaphysis cross sections, in the present study did not show any differences in the radiodensity of these sections. In this technique the thickness of sections, which determines the radio-opacity of the preparations, is quite impossible to control. The section varies in thickness ($100 \pm 10\text{nm}$) as well as there is a possibility of regional thickness variation within the same section. So it is reasonable not to appreciate any differences in radio-opacity of sections between the two groups.

The finding that the cross sectional marrow area is similar in both the groups at all intervals shows that, at the doses used

in this experiment, B-APN does not have any effect on resorption at the endosteal surface. This further eliminates the possibility of influence by resorption on our later experiments. The measurements of the cross sectional cortical area show that equal amount of bone is formed in the B-APN treated chick and in the controls. These findings are consistent with those of earlier authors (Rosenquist et al., 1977, Sandhu and Jande, 1982a).

4.4.6 Effects on Linear Bone Apposition

The findings of the cold tetracycline fluorescent labelling measurements of amount of bone matrix formed, as shown by the width between the two consecutive labels, showed that the linear bone apposition at the periosteal surface of the chick tibia was comparable in both the groups at all intervals during the present experiment.

The cold tetracycline fluorescent labelling has been used in earlier studies to evaluate the effect of B-APN on bone (Berquist, 1960; Berquist and Hulth, 1963). These studies did not clearly indicate any effect of B-APN on bone. Rosenquist et al., (1977) investigated the bone matrix labelling and found no effect of the B-APN on bone matrix formation. The results of the present investigation that B-APN does not affect the amount of bone matrix formed, are similar to that of Rosenquist et al., (1977) and that of Sandhu and Jande, (1982 a).

4.4.7 Effects on ^3H -Proline Incorporation

An analysis of ^3H -proline incorporation, in the present

investigation at different intervals, into tibias of the controls and B-APN treated chicks showed that the bones from both groups incorporated similar amounts of this isotope. As discussed earlier that the ^3H -proline incorporation is a true measure of bone matrix formation, the results of this investigation suggest that B-APN, at the doses used, did not inhibit the synthesis of the bone matrix. These findings are also supported by our finding in this study (as discussed above) that the linear bone apposition and cross sectional cortical area is not different in chicks treated with B-APN and the controls. These findings are also consistent with our earlier investigation on rats (Sandhu and Jande, 1982 a).

4.4.8. Effects on ^3H -Tetracycline Incorporation

The present study showed, on analysis of ^3H -tetracycline incorporation into the bones of the controls and B-APN treated chicks at different intervals, that the incorporation of this isotope is significantly lower in bones of the chicks treated with B-APN as compared to the controls.

It has already been established that the lathyrogen B-APN does not affect the dynamics of tetracycline which could in turn affect its incorporation into bones.

The lower incorporation of ^3H -tetracycline, which has been established as a measure of mineralization, shows a lower deposition of minerals in bones and the data on bone ash contents support these conclusions.

The lower amount of mineral deposition in the lathyritic bones can thus be attributed to a smaller area of matrix undergoing mineralization or to a lower amount of mineral per unit area of the matrix synthesized. The amount of bone matrix formed, as indicated by fluorescent microscopic observations, cross sectional cortical area measurements and ^3H -proline incorporation estimation, is almost similar in the controls and B-APN treated chick bones. The lower incorporation of ^3H -tetracycline thus seems due to a lower degree of mineralization in the newly formed bone matrix.

4.4.9 Comparison of Bone Matrix Synthesis and Its Mineralization

A comparison of the bone matrix synthesis as shown by ^3H -proline incorporation as discussed earlier, and mineralization, indicated by ^3H -tetracycline incorporation in controls (i.e. ratio of 4.0767/1.2452) and B-APN treated chicks (3.433/1.281) at 7 days clearly suggest an inhibition of about 19% in mineralization. This inhibition was about 11% at day 14 and increased to 28% at day 21.

4.4.10 Effects on Serum Calcium Level

The comparable serum calcium level in the controls and B-APN fed chicks, during the present investigation, indicate that the lower mineralization of bones with B-APN treatment is not due to lower availability of calcium; as in case of rickets, but may be due to some other factors involved in the bone mineralization process and which may have been adversely affected by B-APN.

4.5 AN OVERVIEW OF INVESTIGATIONS SO FAR AND POSSIBLE MECHANISM OF B-APN ACTION ON BONE

Our study on rats and chicks has established (as discussed earlier) that B-APN at the respective doses used:

1. does not affect the quantity of bone matrix formed at the respective doses in rats and chicks;
2. inhibits the initial mineralization of bone matrix in rats as well as in chicks;
3. does not influence the bone resorption in chicks and
4. does not affect the serum calcium.

Numerous investigators have studied the effect of lathyrogens on bone (as mentioned in introduction, page 9) and most of the osteopathologies produced by B-APN treatment have been attributed to its inhibition of inter- and intramolecular cross linkages of collagen (Barrow et al., 1974). The collagen cross linkages have also been considered to be a prerequisite for the collagen fibrils to become mineralized (Mills and Bavetta, 1968). Further it has been known since 1977 (Rosenquist et al., 1977) that this lathyrogen affects the bone mineralization. Thus because of this correlation of effect on bone mineralization and inhibition of cross linkages, the mechanism of action of B-APN on bone came to be assumed due to effects of B-APN on collagen cross linkages. However, there is still no direct evidence for such an assumption.

Rosenquist et al., (1977) undertook a detailed morphometric study and found that it was the initial rate of mineralization which was inhibited by B-APN. All other parameters of the bone matrix formation were comparable to the controls. The rationale behind their experiment was that if the collagen cross linkages, which are inhibited by B-APN, are prerequisites for collagen fibrils to become mineralized then there should be an inhibition of mineralization by B-APN. They did find such an inhibition and suggested that reasons for this inhibition of bone mineralization was due to fibre defect, produced by B-APN. However, the cross linkages have been also demonstrated to be inhibited in Vit. D deficiency (Mechanic et al., 1972) but here the initial rate of mineralization was not affected (Baylink et al., 1970). Thus Rosenquist et al., (1977) further speculated that perhaps certain other factors involved in the mineralization process may be behind the B-APN effect on bone.

A conclusive proof of mechanism of action of any agent can only be obtained either by direct evidence or by evaluating all the parameters involved in that particular effect after treatment with the agent. The investigation of the organ or tissue specificity, of the said effect, is also of a critical value.

Recently the role of matrix vesicles in the initial bone mineralization has become of major importance, largely due to extensive research by groups lead by Dr. Anderson in U.S.A. and Dr. Ali in United Kingdom. The matrix vesicles are considered

to be the site of initial mineralization (as discussed in Chapter I, page 15).

— However, some other investigators support the view that the non-collagenous glycosaminoglycans of the bone matrix are responsible for initial mineralization (Pugliarello et al., 1970; Baylink et al., 1972; Shuttleworth and Veis, 1972; Weinstock et al., 1972; Vittur et al., 1972).

In view of the above discussion and without any substantial proof in favour of collagen fibrils as the nidation sites for initial mineral deposits, the B-APN induced inhibition of collagen cross linkages being the sole cause of the inhibition of bone mineralization by B-APN becomes questionable.

The other parameters which could be affected and cause an inhibition of mineralization are the ion transport enzymes such as Ca^{++} , Mg^{++} -ATPase, and alkaline phosphatase related to calcium transport into or away from the bone and those associated with concentration of calcium and phosphates into the matrix vesicles.

4.6 ENZYMES RELATED TO THE PROCESS OF MINERALIZATION

4.6.1 Alkaline Phosphatase

There is a never ending controversy going on about the role of the enzyme alkaline phosphatase in the bone metabolism. In 1923 Robinson proposed that alkaline phosphatase helps to hydrolyse inorganic pyrophosphates (PPi) and thus by raising the local concentration of phosphate promotes the process of the mineralization. This was even called a calcifying factor. The proponents of this theory draw support from the following for a role of alkaline phosphatase in mineralization.

(i) A correlation of alkaline phosphatase with calcium transport

It is known that alkaline phosphatase is present at various sites in the body and there is an active transport of calcium going on at those sites. The presence of alkaline phosphatase in brush border of intestine and proximal convoluted tubules of kidney (Sheila and Lawson, 1978) prompted many investigators to propose a similar role for alkaline phosphatase in bone (Kowarski and Schachter, 1973).

(ii) Alkaline Phosphatase as pyrophosphatase

Fleisch and Bisaz, (1962) and Fleisch et al., (1966) proposed that PPi is an inhibitor of the mineralization and alkaline phosphatase brings about the hydrolysis of PPi and thus acting as pyrophosphatase promotes mineralization. On the other hand Anderson and Reynold (1973) proposed that small quantities of PPi rather promote mineralization. They hypothesized that by

the action of alkaline phosphatase, PPi is hydrolysed, and the released Pi reacts with locally increased concentration of calcium thus leading to an increase in initial mineralization.

(iii) Hydrolysis of phosphate esters by alkaline phosphatase

Anderson, (1976) has proposed another role for alkaline phosphatase. According to him it hydrolyzes the ester phosphates, thus raising the concentration of $Ca \times P$ product and bringing about the formation of apatite crystals.

Notwithstanding the above, many investigators believe that alkaline phosphatase is in fact more closely related to the formation of certain organic components of the bone matrix (Siffert, 1950; Strates et al., 1971; Firschein and Urist, 1971; Dixit, 1972, Francis et al., 1973). In recent years, however, the discovery of matrix vesicles as the site of initial mineralization (Anderson, 1967; Bonucci, 1967) and the presence of alkaline phosphatase on vesicle membranes (Matsuzawa and Anderson, 1971; Bernard, 1978) have changed the balance in favour of the hypothesis that alkaline phosphatase is involved in the process of mineralization. Further Thomas and Rump, (1979) have shown by inhibiting alkaline phosphatase, with levamisol, that this inhibition resulted in reduced rate of the mineralization. Thus although a precise role for alkaline phosphatase in the mineralization is still not agreed upon there is no doubt that this enzyme is involved in the process of mineralization.

4.6.2 Ca^{++} , Mg^{++} -ATPase

Since the discovery of matrix vesicles in cartilage (Anderson, 1967, Bonucci, 1967), and bone (Bernard and Pease, 1969), many researchers have been trying to put forward a model which can explain the role of matrix vesicles in the initial phases of the mineralization. The evidence that an ATPase, which is dependent on divalent cations (Ca^{++} , Mg^{++}), is present on membranes of the matrix vesicles (Anderson, 1970, Matsuzawa and Anderson, 1974) made many investigators look for a role of this enzyme in the process of mineralization. Ali and Evans, (1973) proposed that there is an energy dependent calcium transport from the bone matrix into the matrix vesicles and it is mediated by the divalent cation (Ca^{++} , Mg^{++}) dependent ATPase. They ascribed a role to this enzyme, similar to Na^+ , K^+ -ATPase in Na^+ , K^+ -pump. Certain doubts, however, were expressed about the overlapping substrate specificities of alkaline phosphatase and Ca^{++} , Mg^{++} -dependent ATPase (Felix and Fleisch, 1976) and it was suggested that these are not two different enzymes (Felix and Fleisch, 1974, 1976). Other investigators have recently shown that these are two different enzymes though they might have capabilities of acting on the same substrates (Sela et al., 1978; Guo and Messers, 1978; Skillen and Rehbani-Nobar, 1980).

Messers et al., (1975) and Granstrom et al., (1977) have proposed that Ca^{++} , Mg^{++} -dependent ATPase brings about

the hydrolysis of ATP which has been considered an inhibitor of mineralization or it might act as a pyrophosphatase. Although its precise role is yet to be elucidated, like alkaline phosphatase, most of the investigators agree that it is involved in concentrating the calcium into matrix vesicles.

4.7 CHICK EMBRYONIC BONES AS A MODEL FOR BIOCHEMICAL STUDIES OF B-APN EFFECTS

The biochemical studies on the bone tissue are hindered by the fact that it is quite difficult to prepare and isolate different fractions for various analyses. The embryonic bones provide an ideal model to isolate and prepare various fractions for the above mentioned studies.

The preservation of enzyme activity for histochemical studies of bone is a critical factor. The decalcification with chelating agents, such as EDTA, over a long period of time results in loss of most of the enzymatic activities. The embryonic bones provide an advantage over adult bone that decalcification can be accomplished quickly and thus a major part of the activity can be visualized by histochemical reactions.

Furthermore it is quite easy to fix small pieces of these bones as condensation of the matrix and full mineralization has yet not taken place. Similarly the penetration of the resins used for embedding in embryonic bones is better as compared to the adult bone tissue. The ultramicrotomy of bone is another problem which hinders the workers who want to evaluate with transmission electronmicroscopy certain parameters of bone metabolism. The embryonic bones, because of limited presence of minerals in them, are very easy to cut and thus enables the investigators to see the mineral matrix relationship.

The various stages in the development of chicks, especially

those of bone, are very well documented (Fell, 1925). It is known that the mineralization of tibia and femur in chick embryo start at day 7 of incubation. The events taking place in the development of bone can be caught at any specific stage and effects of any experimental factor on those events can be evaluated. Taking into consideration the above mentioned factors a comprehensive study in chick embryos, evaluating the effect of B-APN on all the parameters of the bone, which has already been investigated in rats and chicks, was undertaken so that the data on the effects of B-APN on the activities of the enzymes could be correlated with the effects on the initial mineralization.

4.8 EFFECTS OF B-APN ON CHICK EMBRYONIC BONE MATRIX FORMATION AND ITS MINERALIZATION AND POSSIBLE MECHANISM OF ACTION

4.8.1 Body Weight and Tibial Length

The present investigation on chick embryos showed that, at the doses used in these experiments, B-APN did not produce any mortality. The body weight of the embryos was also not affected. The length of tibias were comparable in the controls and B-APN treated embryos although hundred percent of the tibias were deformed.

It is clear from the above findings that B-APN, at the doses used, does not affect the general body growth and that of the bones at the organ level. These findings are similar to earlier investigators (Rosenquist et al., 1977) and our own findings in rats and adult chicks (Sandhu and Jande, 1982 a, b).

Although the doses used were not toxic enough to produce 50% mortality rate (LD₅₀) the deformities present in all the tibias indicated that the B-APN has produced some effect on the bones. These deformities of bones may be related to mineralization defect (Dasler and Mosby, 1954; Sandhu and Jande, 1981 a, b) or due to deficiency in collagen tensile strength (Piez, 1968).

4.8.2 Effects on ³H-Proline Incorporation

The analysis of ³H-proline incorporation, as a measure of the bone matrix formation, showed that a comparable amount of ³H-proline incorporated in the bones of the controls as well as B-APN treated chick embryos.

It has been shown that B-APN does not inhibit the matrix synthesis (Golub et al., 1968; Rosenquist et al., 1977). The findings of the present study are in agreement with those of above authors and our own findings in rats and chicks (Sandhu and Jande, 1982 a, b).

4.8.3 Effects on ^3H -Tetracycline Incorporation

In the present study an analysis of ^3H -tetracycline incorporation showed that with B-APN treatment there is a significant inhibition of ^3H -tetracycline incorporation in embryonic bones.

The incorporation of ^3H -tetracycline has already been established to be a measure of initial mineralization (Sandhu and Jande, 1982 a, b). The lower incorporation of the ^3H -tetracycline into the bones of embryos treated with B-APN could be attributed either to a lower amount of matrix formed or to a lower amount of mineral present per unit area of the organic matrix formed. The amount of matrix formed, as shown by ^3H -proline incorporation, is comparable in the controls and B-APN treated embryonic bones. The lower incorporation of ^3H -tetracycline thus can be only due to a lower amount of mineral present per unit area. These findings are similar to those of Rosenquist et al., (1977) and Sandhu and Jande, (1982 a, b).

4.8.4 Effects on Bone Ash Contents


The present investigation showed, on analysis of bone ash contents, that the bones of embryos treated with B-APN had a

significantly lower bone ash-content as compared to the controls.⁹

These findings again point towards a lower amount of mineral present in the B-APN treated embryonic bones as compared to the controls and support the ³H-tetracycline incorporation data (as discussed above). Earlier investigators have also reported similar findings (Rosenquist et al., 1977, Sandhu and Jande, 1982 a, b).

4.8.5. Effects on Enzymes Related to Mineralization

The analysis of activity of alkaline phosphatase and Ca⁺⁺, Mg⁺⁺-ATPase in the membrane fractions obtained from mineralized portions of embryonic tibias showed that the activity of these two enzymes was significantly lower (Alkaline phosphatase P .001; Ca⁺⁺, Mg⁺⁺-ATPase P .001), in bones of B-APN treated embryos as compared to the controls (Table 3.43). As discussed at length earlier, although the role of alkaline phosphatase and Ca⁺⁺, Mg⁺⁺-ATPase in the process of mineralization is not clear, most of the investigators agree that these enzymes play some role in the above mentioned process. It has been clearly demonstrated that the initial mineralization is inhibited by B-APN (Rosenquist et al., 1977, Sandhu and Jande, 1982 a, b). In view of this the inhibition of enzymes alkaline phosphatase and Ca⁺⁺, Mg⁺⁺-ATPase, The enzymes involved in the mineralization (as discussed earlier), by B-APN becomes highly significant with regard to its mechanism of action. (Sandhu and Jande, 1982 c).



4.8.6 Effects On Other Membrane Bound Enzymes

In order to evaluate the effects of B-APN on other membrane bound enzymes the activity of Na^+ , K^+ -ATPase was estimated in the membrane fractions obtained as mentioned above. This analysis showed that activity of Na^+ , K^+ -ATPase was not significantly different in the controls and B-APN treated embryonic bones. (Table 3.4.3).

It is known that Na^+ , K^+ -ATPase is present on the cell plasma membrane and is involved in the transport of Na^+ and K^+ to and from the cell (Glynn, 1962). It is clear from findings of the present investigations that B-APN does not affect the activity of this enzyme. Thus it is quite evident that the inhibition of alkaline phosphatase and Ca^{++} , Mg^{++} -ATPase, by B-APN (as discussed above), is not just part of a generalized inhibition of membrane bound enzymes.

4.8.7 Effects on Acid Phosphatase

In the present study, the analysis of activity of acid phosphatase in bones of the controls and B-APN treated embryos showed that the activity of acid phosphatase is significantly elevated in bones of the embryos treated with B-APN as compared to the controls. (Table 3.4.3).

The enzyme acid phosphatase is a hydrolytic enzyme and is present in a variety of cells including osteoclasts, osteoblasts and osteocytes. The presence of acid phosphatase and other

hydrolytic enzymes in the cells at the surfaces where resorption takes place, the abundant presence of these enzymes in the bone resorptive cells and rise in the activity of acid phosphatase in response to certain hormonal factors which are known to increase bone resorption, has prompted many investigators to ascribe them a role mainly in the bone resorption (Vaes, 1965, 1966, 1968; Doty et al., 1968). It has been further shown that diphosphonates (EDHP and Cl₂ MDPP), which are known to inhibit the bone resorption, depress the activity of the hydrolytic lysosomal enzymes including the acid phosphatase (Morgan et al., 1973; Felix et al., 1976, Fleisch, 1978).

It is generally agreed that the osteoclasts, which have a large number of lysosomes, are very rich in hydrolytic enzymes as compared to other cells like osteoblasts and osteocytes (Holtrop, 1977). The presence of osteoclasts at the resorptive surfaces of bone, in Howship's lacunae, is tantamount to bone resorption.

The presence of acid phosphatase has also been linked with the synthesis of bone matrix (Porter, 1964; Weinstock and Leblond, 1974; Jande and Grosso, 1975). It is clear from our studies on rats and chicks (Sandhu and Jande, 1982 a, b) that bone matrix synthesis is not affected by B-APN. Thus the increase in activity of acid phosphatase with B-APN treatment cannot be due to changes in the matrix synthesis. Another cause of increase in the activity of acid phosphatase can be the toxic effects of

B-APN as it has been shown that acid phosphatase activity might increase in response to certain drugs (Susi et al., 1966; Ellis et al., 1973; Plummer et al., 1975). Thus the elevated acid phosphatase level could either be due to increased resorption or due to the toxic effect on B-APN. The histochemical studies in the present investigation showed the major amount of acid phosphatase to be present in osteoclasts. Thus, it appears that increase in acid phosphatase activity observed in biochemical analysis is due to increase in bone resorption.

4.8.8 Histochemistry

(A) Alkaline Phosphatase

In the present study, the histochemical analysis showed that alkaline phosphatase was present in association with the plasma membranes of osteoblasts and that of the matrix vesicles. In this respect our results are similar to earlier investigators (Matsuzawa and Anderson, 1971; Bernard, 1978).

(B) Acid Phosphatase

The histochemical examination for acid phosphatase showed that the major amount of this enzyme was present in osteoclasts at the endosteal surface of chick embryonic tibias and thus related to resorption. These findings are similar to those reported earlier by previous authors (Schajwicz and Cabrini, 1957; Vaes, 1965, 1966, 1968; Holtrop, 1977).

The ultrastructural localization of acid phosphatase showed that the activity of acid phosphatase was present in relation to lysosomes, golgi vacuoles and saccules.

These observations are similar to the ones reported by earlier investigators (Weinstock and Leblond, 1974; Jande and Grosso, 1975). No appreciable differences in the amount of acid phosphatase reaction in osteoblasts of the controls and B-APN treated materials was observed. These findings correlate with our findings that there is no difference in amount of matrix formed between the two groups.

4.8.9 Tissue Specificity of B-APN Action on Enzymes

In the present investigation to evaluate the organ or tissue specificity of B-APN effects on the enzymes mentioned above, the activities of these enzymes were estimated in embryonic liver tissue as well. The present study showed that activities of enzymes mentioned above are comparable in the controls (controls: Alk. Pase, 506.00 \pm 44.77; Ca⁺⁺, Mg⁺⁺- ATPase, 106.20 \pm 7.28; Acid Phos. 2960.00 \pm 307.65; B-APN : 560.00 \pm 40.43; 104.88 \pm 7.13; 2837.00 \pm 114.7.13) and B-APN treated embryonic liver tissue. (Table 3.4.4).

It is quite clear from the above findings that the effects of B-APN on enzymes mentioned above are bone specific and are not part of a generalized inhibition of all the enzymes in the body.

On the basis of these investigations on chick embryonic bones it is suggested that at the doses used

- B-APN does not inhibit the bone matrix formation;
- it inhibits the initial bone mineralization;

- the mechanism of the inhibition of initial bone mineralization appears to be a bone specific inhibition of enzymes alkaline phosphatase and Ca^{++} , Mg^{++} - dependent ATPase.

4.8.10 Electronmicroscopy

(i) Bone Membrane

The ultrastructural investigations, in the present study, showed no discernible differences in the structure and arrangement of the cells forming a membrane over the periosteal surface of the bones.

The arrangement of osteoblasts in an epithelium like fashion, over the periosteal surface of bones, is very crucial for the maintenance of calcium homeostasis (Terepka et al., 1968; Triffitt et al., 1969; Canas et al., 1969; Newman and Mulryan, 1969). The present investigation showed no morphological effect on this aspect of calcium homeostasis maintenance apparatus with B-APN treatment. Thus it eliminates the possibility of B-APN influence on bone membrane as a possible mechanism of its action on the initial mineralization.

(ii) Osteoblasts and Extracellular Bone Matrix

The major aim of this ultrastructural study was to visualize the nidation sites for initial mineralization and also look for possibility of any alteration in the start of mineralization due to B-APN.

The present investigation showed that B-APN does not have

any effect on various organelles of osteoblasts involved in the synthesis of bone matrix. These findings correlate with our findings, in rats and chicks, that B-APN does not have any effect on the quantity of bone matrix production (Sandhu and Jande, 1982 a, b, c).

Electronmicroscopy of the regions of initial mineralization showed that the matrix vesicles take origin from osteoblasts and as the mineralization front moves toward the cells they accumulate apatite crystals. These findings are similar to those of Anderson, (1969, 1976), Bernard and Pease, (1969), Bonucci, (1970), Rabinovitch, (1974), Ali, (1976).

Ultrastructural study also showed that the initial deposition of minerals takes place in matrix vesicles and not on collagen fibrils. In this respect our findings are consistent with the findings of earlier workers (Bernard and Pease, 1969; Anderson, 1969; Bonucci, 1971; Ornoy et al., 1980). These findings thus make the fibre defect hypothesis, as a cause for B-APN induced inhibition of initial mineralization, questionable.

No differences in number of matrix vesicles or the amount of mineral present in them were detected. These findings are not unreasonable since it is quite difficult to observe and quantitate the minute changes in above parameters by electron-microscopy.

CHAPTER 5

SUMMARY

SUMMARY

* Denotes the original findings of the author.

A. PERIODONTAL LIGAMENT.

1. The molar periodontal ligament and alveolar bone of the rats after feeding them with B-APN (0.25% diet), were examined by microradiography, light microscopy electron microscopy and with histochemistry.
- *2. The microradiographic examination showed a generalized reduction in volume of the alveolar bone, lower radiodensity, widening of medullary spaces, roughening of interdental and interradicular septae and widening of the periodontal ligament space in the rats treated with B-APN as compared to the controls.
3. At the light microscopic level, the cells of the periodontal ligament showed a characteristic arrangement in groups of palisading rows, hyalinization of extracellular matrix, and a very irregular arrangement of collagen fibres in the rats treated with B-APN as compared to the controls. These changes were most pronounced in the region of horizontal and oblique fibres and also in the region of apical fibres.
- *4. The ultrastructural analysis of the regions where the above mentioned effects were the most severe showed that the cells were arranged in groups. Most pronounced change in the ultrastructure of these cells was the vacuolization of their mitochondria. Golgi apparatus and rough surface endoplasmic reticulum were comparable in the controls and the B-APN fed rats.

- *5. The collagen fibrils, in the areas of hyalinization, were arranged in a very haphazard manner and their mean diameter was significantly smaller in lathyrctic periodontal ligament as compared to the controls.
- *6. In histochemical study the activity of acid phosphatase appeared to have increased at the surface of the alveolar bone and in the periodontal ligament of the rats treated with B-APN as compared to the controls.
- *7. The changes in periodontal ligament and the alveolar bone appear to be related to the increased masticatory forces in the wake of reduced tensile strength of the collagen fibrils under the effect of B-APN.

B. RAT BONE MATRIX FORMATION AND ITS MINERALIZATION.

- *8. The effects of B-APN on rat bone matrix formation and its mineralization were evaluated by measuring the incorporation of ^3H -proline, and ^3H -tetracycline, after pulse dosing the rats with two isotopes, and also by measuring the linear bone apposition from topographic deposition of cold tetracycline in the bones.
- *9. At the doses used (0.25% diet) the B-APN did not have any effect on the general body growth as well as the growth of tibiae at organ level.
- *10. The incorporation of ^3H -proline into the bones of rats treated with B-APN was comparable to that of the controls.
- *11. The incorporation of ^3H -tetracycline (a measure of initial bone mineralization) was lower in the bones of rats treated

with B-APN as compared to the controls.

12. The measurements of linear bone apposition, from topographic maps of cold tetracycline administrations, showed a comparable amount of the bone matrix formed in B-APN treated rats and the controls.

13. At the doses used in the experiment the B-APN does not affect the amount of bone matrix formed but inhibits the initial bone mineralization.

C. CHICK BONE MATRIX FORMATION AND ITS MINERALIZATION.

14. Three groups of white leghorn chicks were fed the diets containing B-APN (0.05%, 0.10% or 0.25%). The chicks on the two higher doses showed a severe retardation in body growth and the growth of their bones. So the two higher doses were discarded and the further experiments, on chicks, were conducted with the lowest dose.

*15. The effects of B-APN (0.05%) on bone matrix formation and its mineralization were evaluated by measuring the incorporation of ^3H -proline and ^3H -tetracycline. The bone ash contents and serum calcium were also estimated. The linear bone apposition was measured after administration of cold tetracycline. The measurements of total medullary area and total cortical area, in cross sections of tibia, were made from the microradiographs.

*16. The incorporation of ^3H -proline, total cortical area, total medullary area, and the linear bone apposition were comparable in the controls and B-APN treated chicks.

- *17. The incorporation of ^3H -tetracycline and the bone ash contents were, however, significantly lower in the bones of B-APN fed chicks as compared to the controls.
18. The serum calcium level was comparable in the controls and the B-APN fed chicks.
- *19. At the dose used in this experiment, on chicks, the B-APN does not affect the bone matrix formation and the resorption at endosteum level. However, the mineralization of the bone matrix is significantly lower in the B-APN treated chicks as compared to the controls. The lower mineralization does not seem to be related to a lower availability of calcium for mineralization.

D. INVESTIGATION ON CHICK EMBRYONIC BONES.

20. To elucidate the mechanism of B-APN action on inhibition of the mineralization of the bone matrix further experiments were conducted on chick embryos (12½ days).
- *21. In the first phase of this experiment, the inhibitory effects of B-APN were on first established by measuring the incorporation of ^3H -tetracycline and the estimation of the bone ash contents. The synthesis of bone matrix was measured by the incorporation of the ^3H -proline and was found to be comparable in the controls and B-APN treated embryonic bones.
- *22. The activities of acid phosphatase, alkaline phosphatase, Ca^{+2} , Mg^{+2} -ATPase and Na^+ , K^+ -ATPase were measured in embryonic bone and liver tissues.

23. The histochemical and cytochemical localization for alkaline phosphatase and acid phosphatase was done on glutaraldehyde fixed cryostat sections.
- *24. The activities of alkaline phosphatase and Ca^{+2} , Mg^{+2} -ATPase were significantly lower in the membrane fraction obtained from the bones of B-APN treated embryos as compared to the controls.
- *25. The activity of acid phosphatase, however, was significantly elevated in the sub-cellular particle fraction obtained from the B-APN treated embryonic bones as compared to the controls.
- *26. Activity of Na^{+} , K^{+} -ATPase was comparable in the bones of B-APN treated embryos as well as the bones of the controls.
- *27. The enzymatic assays on embryonic liver tissue showed that the activities of enzymes mentioned above were comparable in the B-APN treated and the control embryos.
28. The histochemical and cytochemical localization of acid phosphatase showed it to be present in osteoclasts and the lysosomes of the osteoblasts.
29. The alkaline phosphatase was present on the plasma membranes of the osteoblasts and the matrix vesicles in the extracellular matrix.
- *30. The ultrastructural analysis of the bone membrane lining the periosteal surface of embryonic tibia showed no differences in the structural arrangement of the osteoblasts and the open channels between them.
- *31. The ultrastructural examination of the cells forming the bone

matrix and the zones of the initial mineralization showed no significant differences, between lathyritic embryonic bones and the controls, in the cells and the structures related to the initial mineralization of bone.

- *32. The inhibition of initial mineralization by B-APN seems to be due to bone specific inhibition of enzymes alkaline phosphatase and Ca^{+2} , Mg^{+2} -ATPase.

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LIST OF PUBLICATIONS

1. Sandhu, H.S. and Jande, S.S. (1978) B-APN induced ultrastructural changes in the periodontal ligament. Anat. Rec. 190: 529 (Abst.).
2. Sandhu, H.S. and Jande, S.S. (1979) Effect of B-APN on formation and mineralization of bone matrix. Anat. Rec., 193: 674 (Abst.)
3. Sandhu, H.S. and Jande, S.S. (1981) Effects of B-APN on rat bone matrix formation and its mineralization. Calcif. Tiss. Intl. 34. 80-85.
4. Sandhu, H.S. and Jande, S.S. (1981) B-APN induced inhibition of bone mineralization, a possible mechanism of action. Anat. Rec. 199 223A.
5. Sandhu, H.S., and Jande, S.S. (1981) A Morphometric and Radioisotopic evaluation of the effects of B-APN on bone matrix formation and its mineralization. Acta Anat. (In press).
6. Sandhu, H.S. and Jande, S.S. (1981) Effects of B-APN on formation and mineralization of bone matrix in chicks. Proc. Can. Fed. Biol. Soc. 24: 230.
7. Sandhu, H.S., and Jande, S.S. (1981) Investigations of alkaline phosphatase, Ca^{+2} -ATPase and Na^+ , K^+ -ATPase during B-APN induced initial bone mineralization inhibition. Acta Anat. (In press)
8. Sandhu, H.S. and Jande, S.S. (1981) Biochemical, Histochemical and Microradiographic evaluation of B-APN effects on chick epiphyseal plates. Anat. Rec. (In press).

ABSTRACT

Effects of Beta-aminopropionitrile (B-APN) on Periodontal Ligament (PDL) and alveolar bone of rats were evaluated by microradiography, light microscopy, histochemistry and electron microscopy.

Also the effects of B-APN on bone matrix formation, its mineralization and to find a possible mechanism of B-APN action on bone were investigated in rats, young adult chicks and chick embryos (12½ days). The techniques of microradiography, fluorescent microscopy after cold tetracycline labelling, bone ash content measurement, serum calcium estimation and liquid scintillation after pulse dosing the experimental animals with ^3H -proline and ^3H -tetracycline, were used. In chick embryos in addition to the above techniques biochemical assays were done to determine the activities of alkaline phosphatase, Ca^{++} , Mg^{++} -ATPase, acid phosphatase and Na^+ , k^+ ATPase in bone as well as liver tissues. Activities of alkaline phosphatase and acid phosphatase were further localized at the light microscopic level (Burstone's method) and at the ultrastructural level (Gomori method). The bone membrane and the zone of initial mineralization, in chick embryos, were examined at the ultrastructural level.

The Periodontal Ligament of rats, after 28 days of B-APN feeding, showed hyalinization of the extracellular matrix, loss of a regular arrangement of collagen fibrils, and the arrangement

of fibroblasts in palisading groups. At the ultrastructural level, the cells showed vacuolization of mitochondrial matrix. The collagen fibrils showed a haphazard arrangement and a significantly smaller diameter in B-APN fed rats as compared to the controls.

The alveolar bone, in microradiographs showed a lower density, widening of nutritive and marrow spaces and a general reduction in bone volume. The histochemical examination at the light microscopic level showed the presence of increased acid phosphatase activity in B-APN fed rats as compared to the controls.

During three weeks of B-APN feeding to rats the general body growth and the growth of bones at the organ level were not affected. Although the linear bone apposition and the incorporation of ^3H -proline were not significantly different between B-APN fed and the controls yet, the incorporation of ^3H -tetracycline was significantly lower in B-APN fed rats.

In a similar study on chicks, after three weeks of B-APN feeding, the general body growth and the growth of bones at the organ level were unaffected. The incorporation of ^3H -tetracycline and bone ash contents were significantly lower in B-APN fed chicks as compared to the controls. However, the linear bone apposition, total cross sectional cortical and medullary areas in tibias, serum calcium level and the incorporation of ^3H -proline were comparable in B-APN fed chicks and controls

during all intervals of the experiment.

After four days of B-APN treatment, the chick embryos, showed significantly lower incorporation of ^3H -tetracycline and a lower bone ash content, however, the body weight, tibial length and the incorporation of ^3H -proline were comparable in the controls and B-APN treated embryos.

The biochemical investigation of enzymes showed significantly lower activities of alkaline phosphatase and Ca^{++} , Mg^{++} -ATPase in B-APN treated embryonic bones as compared to the controls. The activity of acid phosphatase was significantly higher in the bones of B-APN treated embryos as compared to the controls while there was no effect on activity of Na^{++} , K^{+} -ATPase. The enzymatic assays on embryonic liver tissue showed that the activities of all above mentioned enzymes were comparable in the controls and B-APN treated embryos.

The histochemical and cytochemical studies showed alkaline phosphatase to be present on osteoblast plasma membrane and on the matrix vesicles while the acid phosphatase was present in osteoclasts and the lysosomes of the osteoblasts.

The ultrastructural study of bone membrane on the periosteal surface of embryonic tibias and the zones of initial mineralization showed a comparable structural arrangement of bone membrane and structures involved in mineralization in the control and B-APN treated tissues.

On the basis of the above mentioned findings it is concluded that,

- (i) the effects on the cells and fibres of periodontal ligament are secondary to the reduced tensile strength of collagen fibrils due to B-APN treatment;
- (ii) The collagen fibrils are of smaller diameter due to the inhibition of inter and intra molecular cross linkages by B-APN . . .
- (iii) B-APN seems to produce a condition in alveolar bone which appears similar to systemic periodontal disease i.e. periodontosis.
- (iv) at the doses used, in rats; chicks and chick embryos, B-APN does not affect the amount of bone matrix formed.
- (v) The initial mineralization of the bone matrix formed is inhibited by B-APN.
- (vi) The structure and integrity of the bone membrane and other structures in the pre-osseous zone do not appear to be affected by B-APN.
- (vii) The inhibition of mineralization seems to be due to bone specific inhibition of the enzymes alkaline phosphatase and Ca^{++} , Mg^{++} -ATPase by B-APN.
- (viii) The elevated acid phosphatase activity seems to be due to the toxic effects of B-APN.