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AN AUTORADIOGRAPHIC STUDY OF LATHYRISM USING TRITIATED THYMIDINE

by

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This dissertation submitted by Dwayne A. Ollerich in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the University of North Dakota, is hereby approved by the Committee under whom the work has been done.

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CHAPTER I

INTRODUCTION

The term "lathyrism" was originally used by Cantani in 1873 to describe a disease seen in humans and domestic animals that had ingested large quantities of seeds from certain pulses of the genus Lathyrus (Cited from 1.) Mention of such a disease has also been found in the writings of Hippocrates and Pliney and Elder (Cited from 1.) The disease in humans is usually manifested as a neurological disorder resulting in spastic paralysis of the lower extremities. Human lathyrism is endemic to countries near the Mediterranean Sea and to India and parts of Russia (2). Three species, Lathyrus sativus, Lathyrus cicera and Lathyrus clymenum have been implicated in the etiology of human lathyrism. These legumes grow well even under poor crop conditions and during periods of famine Lathyrus seeds often make up a large portion of the poorer peasants' diet. Attempts at identifying the toxic factor of these seeds have been relatively unsuccessful. However, Bell (3) has recently isolated the neurotoxic amino-acid, alpha-gamma-diaminobutyric acid, from twelve species of Lathyrus seeds. He was not able to isolate this amino acid from seeds of the three species suspected of producing the human disease. He did find that seeds of these three species contained major quantities of a new natural amino acid identified as homoarginine. Thus, no known neurotoxic factor has, as yet, been isolated from the seed of Lathyrus sativus, Lathyrus cicera or Lathyrus clymenum. A

neurotoxic amino-acid, beta-cyanoalanine, has been isolated from Vicia sativa and Vicia augustafolis (4). These leguminous weeds are closely related to plants of the genus Lathyrus, and seeds of Vicia sativa have been found mixed with seeds of Lathyrus sativa which were to be used for human comsumption (4). In 1933 Geiger, Steenbock and Parsons (5) found that white rats fed a diet containing over 25 percent Lathyrus odoratus seeds developed lesions of the skeleton and other mesodermal tissue. They reported lameness, spinal curvature, sternal curvature, enlargement of costochondral junctions, hernias and malformation and abnormal coloring of long bones. Robinson and Bast (6) soon confirmed these findings and also initially described exostoses that developed on the surface of long bones in affected rats. The exostoses appeared as rapidly growing bony masses composed of proliferating osteogenic tissue beneath the periosteum of long bones. A high incidence of dissecting aneurysms in affected rats was first reported by Ponseti and Baird (7). These lesions could not be produced in rats when the feeding of the diet was started later than 51 days of age. In 1955 Ponseti and Shepard (8) noted loosening of ligamentous and tendinous insertions and lesions of epiphyseal plates in lathyric rats. They felt that the latter two defects resulted in the following abnormalities: metaphyseal tuberosity defects, degeneration of the intervertebral discs, intervertebral disc herniation, epiphyseal slipping, kyphoscoliosis, thoracic deformities, valgus of the forepaws, subluxations and dislocations of the shoulder, diastasis of the sacro-iliac joints and formation of periosteal new bone. They also pointed out the similarity of these lesions to certain spontaneous human skeletal dis-

eases.

Selye (9) has subsequently pointed out that there should be a distinction made between the mesenchymal disease of experimental animals and the neurological disease of humans. He suggested the term "neurolathyrism" to designate the disease in humans, and the term "osteolathyrism" to designate the disease as seen in experimental animals. Generally, however, any toxic effects caused by the ingestion of Lathyrus seeds, the toxic factor of these seeds, or chemical compounds closely related to the toxic factor, have been called "lathyrism". The latter statement designates the manner in which the term "lathyrism" will be used in this paper.

The production of an apparent generalized disease of mesenchymal tissue in the rat led several investigators to attempt to determine the toxic factor of Lathyrus odoratus seeds. McKay et al. (10) were the first workers to isolate a lathyrogenic substance from seeds of Lathyrus odoratus. The compound had an apparent empirical formula of $C_8H_{13}O_3N_3$. Schilling and Strong (11) identified the product as beta-(L-glutamyl)-aminopropionitrile and subsequent investigations (12, 13) showed that the active part of the molecule was beta-aminopropionitrile (BAPN). Structurally related compounds, such as aminoacetonitrile (AAN) (13), beta-mercaptoethylamine (12), semicarbazide (14), methylene-aminoacetonitrile (MAAN) (15), bis (beta-cyanoethyl)-amine and beta-beta prime-iminodipropionitrile (16), also produced lathyric changes. Aminoacetonitrile (AAN) is apparently the most toxic of all the lathyrogenic agents.

Experimental lathyrism offers an excellent model from which to study connective tissue pathology. Thus, numerous investigators have studied the effects of a variety of lathyrogenic agents on rats, mice, rabbits, chickens, turkeys, guinea pigs, fish, toads, salamanders, tadpoles and dogs. Because of the variety of lathyrogenic agents and experimental animals used, there is a great deal of confusion in the literature regarding the precise action of lathyrogenic agents. It is generally agreed, however, that both the mucopolysaccharide and fiber fractions of connective tissue ground substance are somehow affected. There are also reports of connective tissue cell hyperplasia at most sites of lathyric lesions. In addition, it is very difficult to interpret the changes noted in lathyric animals without assuming some metabolic derangement of the connective tissue cells. Some evidence has also been presented that demonstrates an involvement of other organ systems in experimental lathyrism. The literature concerning these five areas will now be reviewed.

I. Lathyrogenic Effect on Mucopolysaccharides

Early reports in the literature indicated an increase in the quantity of mucopolysaccharides present in the ground substance of lathyric connective tissue. Churchill et al. (17), Pyorala et al. (18), Menzies and Mills (19) and Gillman and Hathorn (20) have all reported an increase in metachromatic material in the aortic lesions of lathyric rats. Churchill et al. (17) concluded that the mucopolysaccharide increase was predominately in the chondroitin sulfate B fraction on the

basis that metachromatic staining did not decrease after treating the sections with hyaluronidase. Concomitant with the ground substance increase they noted elastic fiber degeneration. In contrast to this work, Pyorala et al. (18) found a decreased metachromasia after hyaluronidase treatment, and according to Menzies and Mills (19) elastic lamina degeneration began only after fibroblasts had infiltrated the aortic wall. Gillman and Hathorn (20) have suggested that the aortic rupture might be due to some derangement of the balance between lysis and regeneration of the elastic membranes based on some failure in the mucopolysaccharides of the ground substance. A decrease in metachromatic material in the walls of major arteries of long term lathyric rats has also been reported (21).

Changes in mucopolysaccharide staining have also been noted in the healing wounds of lathyric rats (22), where there was an accumulation of PAS positive material, and in the epiphyseal plates of lathyric animals (8, 18, 23, 24.). Ponseti and Shepard (8), who first described histological changes in lathyric epiphyseal plates, also mentioned changes in the usually homogenous metachromatic staining. Ramamurti and Taylor (23) found non-metachromatic linear areas in the matrix of lathyric epiphyseal cartilage. Many of these linear areas stained with the PAS reaction. Rents or tears appeared in the linear areas in the zone of maturing cartilage cells. The lesions in the epiphyseal cartilage regressed dramatically after removing BAPN from the diet. Karnovsky and Karnovsky (24) have stated that these histochemical findings should be cautiously considered since the increase of mucopolysaccharide

staining intensity could reflect an increase in the number of anionic groups available for binding of the dye.

Since histochemical methods do not demonstrate a quantitive change in mucopolysaccharides of lathyric connective tissues, some investigators have used biochemical methods using hexosamine and uronic acid as an index of mucopolysaccharide content. In addition, radiosulfate has been used to demonstrate quantitative changes in the sulfated mucopolysaccharides. Results from these methods have also been conflicting.

Grant, Hathorn and Gillman (25) found increased hexosamine and uronic acid in lathyric rat aortas but no changes in hexosamine content of skin, tail tendon,or femur. Burzynski (26) noted an increase in synthesis of of protein bound hexose in cultures of strain L fibroblasts. This observation supported other observations of an increase in mucopolysaccharide content in the connective tissue ground substance of experimental lathyric animals. Mielke, Lalich and Angevine (27) found no significant change of hexosamine content of connective tissue developing in croton oil pouches of lathyric rats. Karnovsky and Karnovsky (24) also found no significant difference in the hexosamine content of lathyric epiphyseal cartilage, but they did find a decreased uronic acid content. On the other hand, Castellani and Castellani-Bisi (28) found a 36 percent decrease of hexosamine in lathyric rabbit epiphyseal cartilage. The latter finding has been somewhat substantiated by Pedrini and Pedrini-Mille (29) who found that AAN-treated rabbits synthesized only one-fourth as much hexosamine as the epiphyseal plates of control animals.

Karnovsky and Karnovsky (24) found that the uptake of radiosulfate by

lathyric epiphyseal cartilage was markedly decreased two days after starting lathyrogenic treatment. Shintani and Taylor (31) demonstrated in Vitro decrease in radiosulfate uptake by lathyrogen-treated epiphyseal cartilage. A decreased uptake of radiosulfate by the long bones of AAN-treated chick embryos has also been noted (32). The effect of a lathyrogen on radiosulfate uptake of perfused rat liver apparently depends upon when the liver is exposed to the lathyrogenic agent (33). When the lathyrogen was given to the rat before perfusion of the liver, there was less incorporation of S^{35} into the undializable fraction of perfusate plasma. However, when the lathyrogen was given in the perfusate, the incorporation of S^{35} was equal to or greater than control values. Increased radiosulfate uptake was also found by Bauer and Carlsson (34) in femur shafts of BAPN-treated rats, by Belanger (35) in the epiphyseal plates of lathyric rats and chicks, and by Bickley and Orbison (36) in cultures of the strain L fibroblasts. Utilizing data gained from autoradiographic studies, Kennedy and Kennedy (37) have concluded that there was no inhibition of the cellular utilization of S^{35} sulfate or abnormal accumulation of labeled sulphated-mucopolysaccharides in lathyric lesions. There was, however, abnormal distribution of the labeled compound, especially in epiphyseal cartilage. Additional studies on the uptake of labeled amino acids led them to suggest that lathyrogenic agents act outside the cell by preventing complex formation between protein and chondroitin sulfate A and C, thus causing a defect in fiber formation. According to them this would also account for the weakening of collagen fibers and the

reactive cellular hyperplasia that will be described later.

II. Lathyrogenic Effect on Collagen

One of the early postulations was that the defect in lathyrism was a disturbance in collagen metabolism, since tissues high in collagen content are primarily affected (38). There is ample evidence in the literature to substantiate this postulation since numerous authors have noted a marked increase in the extractable collagen fraction of lathyric connective tissues (39-48). The increase in extractable collagen is apparently accompanied by a parellel rise in hydroxyproline excretion (45,47). However, it is not definitely known if the increase in extractable collagen is caused by the direct action of lathyrogenic agents. Recent evidence indicates that the synthesis of collagen is not impaired. The defect appears to be on a molecular level. It has been proposed that the large pool of extractable collagen is derived from old insoluble fibers transformed to an extractable state (39,40). Electron microscope studies on BAPN-treated chick embryo skin have also given this impression (41). Collagen is present in fibrillar form in lathyric chick-embryo skin, but extraction with cold 1 molar saline causes a dissolution of the filaments to fine fibrils of varying diameters. The authors concluded that lathyrogenic agents induce disruption of intermolecular cross-linking within normally insoluable collagen fibers, allowing them to dissolve in cold neutral salt solutions.

An opposite view is that the large soluable collagen pool is due to lathyrogenic prevention of mature collagen formation from newly synthesized collagen (31,40,49). Experimental evidence indicates that this block may

be due to failure of formation or disruption of intramolecular cross-bonding, which is an important step in collagen fiber maturation (50,51). Gross (52) states that both intermolecular and intramolecular cross-links may be affected.

Recent evidence indicates that there may be some substance to both of the views just discussed. Tanzer and Gross (53) have found that only a portion of the extractable collagen from lathyric chick embryos represents newly formed protein and they proposed that the lathyritic process affects collagen in all states of aggregation.

In an attempt to determine the precise manner in which lathyrogens affect collagen, Levene (54) has advanced the hypothesis that lathyrogenic agents act by blocking carbonyl groups on the collagen molecule, thus preventing normal cross-linking. However, Orloff and Gross (55), using C^{14} labeled BAPN, found less than one molecule of BAPN per 100 molecules of protein from highly purified lathyric chick bone collagen. This ratio was not high enough to block carbonyl groupings. They suggested that the error caused by lathyrogens is probably metabolic.

III. Lathyrogenic Effect on Connective Tissue Cell Proliferation

Proliferation of connective tissue cells at the site of lathyric lesions has been reported by many authors. The initial report on developing aortic aneurysms by Ponseti and Baird (7) stated that an attempt to repair the defect in the aortic wall was made with the appearance of a very cellular connective tissue. Bachhuber and Lalich (56) noted a proliferation of fibrous connective tissue in the intima, media and adventitia, which they also interpreted as an attempt at

repair of the lesion. Walker and Wirtschafter (57) thought that the aortic aneurysms developed as the result of two inseparable processes, destructive elastolysis accompanied by reparative fibroblastic proliferation. They also noted the destructive elastolysis and fibroblastic proliferation throughout the coronary and pulmonary arteries. More extensive changes were described by Walker (21), who found elastic lamina involvement and fibroblastic proliferation in almost all major arteries of young lathyric rats. Merkow, Lalich and Angevine (26) also noted the relationship of fibroblastic proliferation and elastic fiber destruction. However, they associated the disappearance of the elastic fibers with the onset of fibroblastic proliferation around areas of hemorrhage. From these findings it appears that the majority of authors investigating lathyric aortic lesions consider the fibroblastic proliferation as an attempt at repairing previously formed lesions.

As mentioned previously, Robinson and Bast (6) first described the rapidly growing exostoses that develop on the long bones of lathyric rats. They suggested that the pull of spastic muscles on the periosteum might be the stimulus for periosteal cell proliferation. Hamre and Yeager (58,59) have demonstrated that muscle tension is indeed necessary if exostosis formation is to occur. When the pectineus and adductor longus muscles, or the nerves supplying these muscles, are sectioned the exostosis that usually forms at this site does not develop. Menzies and Mills (19) stated that the fibroblastic proliferation in the periosteum was obviously reactive and suggested that a change in the ground substance could be the stimulus that led to cellular proliferation and the production

of osteoid matrix and bone. Gardner, Dasler and Weinmann (60) have described the masticatory apparatus of lathyric rats. They found exostoses developing at the sites of muscle attachment on the cranium and mandible as early as one week after beginning the experimental diet. The proliferating connective tissue assumed almost neoplastic qualities, invading muscle tissue and leading to degeneration of muscle cells. They also regarded the proliferation as a reactive phenomenon secondary to changes in the cementing substance. Kennedy and Kennedy (36) also believe that the periosteal proliferation noted at the site of muscle insertion is a reactive response. However, they feel that the proliferation is secondary to weakening of the fibrillar elements in the area.

On the other hand, Yeager and Hamre (61) found an increased number of mitotic figures in the periosteum at the site of adductor longus-pectineus insertion in lathyric rats after only twenty-four hours on a lathyrogenic diet. This indicated to them that the response of this area to the lathyrogen was practically immediate and was manifested as a stimulation of the periosteal fibroblasts. Other segments of the periosteum not related to muscle insertions failed to respond to the lathyrogenic diet. Bergquist and Hulth (62), however, have reported that bone was deposited generally on the humerus and femur both around and along the entire length of the shaft in AAN-treated weanling rats. The difference in results between the two last papers is probably due to the age of the experimental animals, the length of time on experiment and to the difference in toxicity of the lathyrogenic agents used. The work of

Bergquist and Hulth (62) does indicate that periosteum in general may be affected by AAN and it adds strength to the theory that lathyrogens may directly stimulate connective tissue cells.

Ponseti and Alu (63) and Storey and Varasdi (64) have investigated the effect of AAN on fracture repair in rats. Both groups found excessive formation of poorly formed fibrous tissue, cartilage and bone. At four days the periosteum adjacent to the fracture was increased in thickness due to proliferation of periosteal cells. It was felt that the proliferation of periosteal cells may have been caused by stress due to poor callus formation (64).

Bois and Belanger (65) recently investigated the mitotic activity of epiphyseal cartilage in hypophysectomized animals treated with AAN and somatotropin. Epiphyseal cartilage cells that were preparing for mitosis were labeled by injecting H^3-labeled thymidine. They found that when hypophysectomized rats were injected with AAN there was actually a slight decrease in the labeling index. The mitotic rate was significantly increased when the rats were treated with both growth hormone and AAN. They concluded that lathyrism produced by AAN is dependent upon the presence of growth hormone and is partly manifested by increased mitotic activity of the cartilage cells.

The effect of lathyrogenic agents on wound healing in rats has also been studied : Enzinger and Warner (22) reported evident fibroblastic proliferation in the wound area after AAN treatment for fifteen days. They started lathyrogenic treatment twenty-four hours after wounding. Krikos and Orbison (66) found that the granulation tissue was thinner and the number of fibroblasts per unit area was decreased. However, they

did note fibroblastic proliferation in the cut edge of the panniculus carnosus. They wounded their animals nine days after starting BAPN in the diet.

Levy (67) has noted tumor-like growths on the notochord of fish treated with a lathyrogen. These tumors developed a short time after the removal of the inciting agent. Since the tumors did not regress with age, it is possible that they are the result of a direct lathyrogenic stimulation of the cells.

Other cells reported to increase in number are megakaryocytes (68), reticuloendothelial cells (68) and mast cells in the bone marrow of lathyric rats (69,70,71).

On the other hand, there are reports in the literature that indicate that lathyrogens may actually inhibit fibroblastic proliferation under certain circumstances. Enzinger and Warner (72) failed to note excessive fibroblastic proliferation in connective tissue developing in polyvinyl sponges that had been implanted in adult lathyric rats. Bickley (73) has recently reported that proliferation of strain L fibroblasts in suspension cultures is decreased in approximately inverse proportion to the concentration of BAPN. Fiume (74) has found that AAN inhibits histiocytic and fibroblastic proliferation in the liver of rats that had been exposed to toxic doses of carbon tetrachloride.

IV. Lathyrogenic Effect on Connective Tissue Cell Metabolism.

Many investigators have felt that the severe lesions of lathyrism could be explained only if the lathyrogen caused a disturbance in the

basic metabolism of the connective tissue cell. For this reason the metabolic distribution of lathyrogens has been investigated. In addition, many workers have studied the effects of lathyrogenic agents on enzymes and on trace minerals in relationship to their effect on the lathyrogenic process.

The general distribution of C^{14} labeled AAN in the rat has been studied. Ponseti et al. (75)found that the major portion of the radioactivity was eliminated in a very short time. Some radioactivity appeared in the respiratory carbon dioxide. Liver protein contained high initial C^{14} concentrations. Liver lipid, liver glycogen and the sulfated mucopolysaccharides of epiphyseal plates also contained some radioactivity. C^{14} label was noted in urinary creatine, allantoin and hippuric acid, but the major urinary fraction containing C^{14} could not be identified. The major metabolic product of BAPN was found to be cyanoacetic acid (76). This compound does not cause lathyric changes itself, but rather appears to be a detoxification product of BAPN. When a 24 percent casein diet was given to lathyric rats, cyanoacetic acid excretion increased and the severity of the lathyric deformities decreased (77). This finding led Merkow, Lipton and Lalich (77) to propose that the animals on high casein diets were better able to detoxify BAPN.

Some investigators have attempted to determine the effect of lathyrogenic agents by studying the changes in enzyme systems and in other metabolically active components of the affected cells. Karnovsky (78)

found a marked decrease in glycogen content of cells in lathyric epiphyseal plate. The decrease in glycogen appeared to be independent of adrenal activity, reduced dietary intake and failure to grow. Concomitant with the cellular glycogen decrease, there was a decrease in histochemically demonstrable phosphorylase. In addition, he found that glycogen synthesis by normal epiphyseal cartilage cells was inhibited by the presence of lathyrogenic agents in an _in vitro_ anerobic system.

The aerobic glycolytic cycle has also been investigated in lathyric animals. Wynn and Ball (79) and Sether (80) investigated succinic dehydrogenase activity in lathyric rats. No changes from normal were observed. These findings do not preclude a block at some other site of the aerobic cycle. Kuhlman (81) found a striking depression of lactic and glucose-6-phosphate dehydrogenase activity in the area of hypertrophic cartilage cells and primary spongiosa of lathyric rabbit epiphyseal plates. Phosphoglucosisomerase was reduced in all three areas of the epiphyseal plates. He felt that the morphological disruption of epiphyseal plates seen in lathyrism is the result of reduction in activity of several enzyme systems normally providing metabolic intermediates and high-energy phosphate necessary for tissue synthesis. Clemmons and Jackson (82) have reported that AAN decomposes with the formation of a substance that is a potent inhibitor of cytochrome oxidase. However, MAAN did not inhibit cytochrome oxidase. Severson (83) histochemically studied the effect of BAPN on cytochrome oxidase in the adductor longus and pectineus muscle and in periosteal cells at the site of insertion of these muscles. He found no inhibition of cytochrome oxidase activity. It is of interest to note that cytochrome

oxidase is a copper containing enzyme and that traces of copper ion are required in the culture medium of fibroblasts before any cytotoxic activity of AAN is noted (84). In turkeys, the symptoms of copper deficiency and BAPN toxicity are similar (85). However, the addition of copper to the diet does not completely prevent the toxic effect of BAPN.

Yeager and Gubler (86) have studied the protein histochemistry of lathyric rat periosteum and epiphyseal plate. No difference from normal was noted in protein sulfhydryl, disulfide and amino groups when similar elements of the periosteum were compared. The RNA content of periosteal cells in normal and lathyric periosteum has also been studied histochemically (87). The early stages of exostosis formation were marked by a change in periosteal cell morphology and a decreased RNA staining intensity. As bone began to form in the inner layer of periosteum, it was found that RNA-staining intensity increased progressively as periosteal cells differentiated into osteoblasts and then decreased when osteoblasts became osteocytes. No apparent inhibition of RNA synthesis was noted. However, Hayatsu and Ukita (88) have found that semicarbazide (a lathyrogen) affords a specific reaction with cytidine residue in ribonucleic acid. This finding is of importance since it is the RNA of the cell that guides cellular protein synthesis and any alteration in the ribonucleic acid might very well be reflected in abnormal protein synthesis.

V. Lathyrogenic Effect on other Organs

Changes in the adrenal glands, thymus, liver, spleen, thyroid, lymphnodes and testicles have been noted in experimental lathyrism.

Dasler (89) found that adrenal weights in male lathyric rats were significantly greater than those of control animals while adrenal weights of female lathyric rats did not differ significantly from controls. However, adrenal weights per unit of body weight were significantly higher for both male and female lathyric rats than for control rats. He felt that this effect might be secondary to the mesenchymal involvement or result from stress. Diaz, Vivanco and Pleguezuelo (90) and Aschkanasy (91) also reported an increase in adrenal weights of lathyric rats. Aschkanasy (91) also reported that the hypertrophy of the adrenal glands went "hand-in-hand" with atrophy of the thymus.

Increased size and weight of liver (91), thyroid (91,92,93), spleen (91) and lymph nodes (91) has also been reported. Mild congestion and depletion of reticuloendothelial cells has also been reported in the spleen (94). Another group of workers found a reticuloendothelial cell hyperplasis in the spleen (68).

The testicles of male lathyric rats was found to decrease in weight while the weights of lathyric rat aorta, heart, kidney and pituitary body did not change (90). However, there may be some renal involvement since Tuominen and Kulonen (95) have found a loss of tyrosine, sulfur-containing amino-acids and histadine from plasma in experimental lathyrism. They attributed the loss to a renal cause.

The recent reports of Fiume (74,96) have added a new dimension of interest and importance to the study of experimental lathyrism. He found that AAN not only inhibited histiocytic and fibroblastic proliferation in the liver of carbon tetrachloride-treated rats, but

also tends to protect the liver cells against the toxic effects of carbon tetrachloride. In addition, he has shown that AAN prevented the inhibition of protein synthesis which occurs in the liver when rats are given dimethylnitrosamine.

From the literature review it is apparent that there are divergent views concerning the primary effect of lathyrogenic agents. It can not be denied that collagen and mucopolysaccharides are altered in lathyrism, but the precise alteration is not known. It has been suggested that lathyrogens act outside the cell by altering the extracellular material, which in turn acts as a stimulus for reactive fibroblastic proliferation (37). There is no direct evidence in the literature to support this theory. In addition, it is difficult to interpret the changes in mucopolysaccharides and collagen without considering some direct action by lathyrogens on the connective tissue cells, since the cells are responsible for the normal maintenance of the intercellular material. There is evidence in the literature that suggests an early increase in mitotic activity of lathyric periosteum (61), which could indicate that the lathyrogen directly stimulated the periosteal cells to begin proliferation. It has also been reported that the effect of AAN on rat epiphyseal plates is partially manifested as an increase in mitotic activity (65). There is also evidence in the literature that a lathyrogen might actually depress connective tissue proliferation in certain circumstances (72,73,74). Moreover, the change in weights of various other organs in experimental lathyrism indicates the possibility of cellular hyperplasia, although the change in weight could be due to an increased water content, as has been found in some lathyric tissues (97,98).

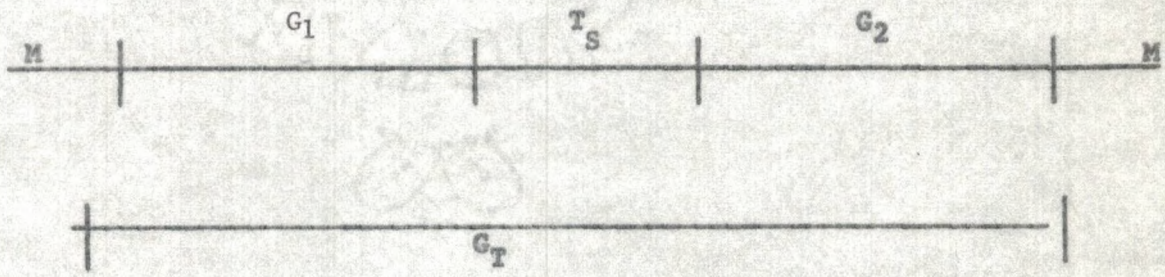
Thus, the possibility exists that lathyrogenic agents may exert their action by affecting cell division in some manner. If this is the case, lathyrogens must exert their influence on the cells very early in the development of lathyrism since early changes have been reported in the intercellular material (49,99). In order to learn whether cells or intercellular material is affected first, it is necessary to know when and how each is affected. Therefore, it is the purpose of this paper to investigate the early effect of a lathyrogen on cell proliferation at the site of lathyric lesions as well as in the adrenal glands that have been reported to increase in weight in lathyric animals.

One method of determining the proliferative rate of a cell population is the use of tritiated thymidine (H^3TDR) coupled with autoradiographic techniques. Labeled thymidine has been shown to be a specific precursor of deoxyribonucleic acid (DNA) (100,101). In all but a few circumstances (102), it is taken up only by cells preparing for division (103). The nuclei of the cells taking up H^3TDR will become radioactive and can be recognized by autoradiography. Tritium emits a very low energy beta particle (0.018 MEV maximum) with an average range of about 1.5 micra (104). Consequently, excellent autoradiographic resolution can be obtained. Another distinct advantage of H^3TDR is the very short period of time that it is available to DNA-synthesizing cells (105). In addition, it has been shown that the tritium is in a stable configeration on the thymidine and that thymidine does not exchange after its incorporation into DNA (Cited from 106). Thus, once the cells are labeled, the thymidine becomes a permanent marker and the radioactivity

is diluted only by mitotic division. H^3TDR is, therefore, a very desirable isotope to use in autoradiographic studies of cell proliferation.

There are several techniques that have been used to extract information from H^3TDR-labeled cell populations. For example, Leblond, Messier and Kopriwa (107) have used H^3TDR as a tool to study the renewal of cell populations. H^3TDR and autoradiography have also been used to study cell migration (108) and the origin and fate of various cell types (109). In addition, much information concerning rate of cell proliferation and the cell generative cycle has been gained from autoradiographic study of H^3TDR-labeled cell populations. Some of the techniques of extracting information from this type of a study will be discussed below.

Lajtha, Oliver and Ellis (110) have divided the generative cycle into four periods (Text Figure I).

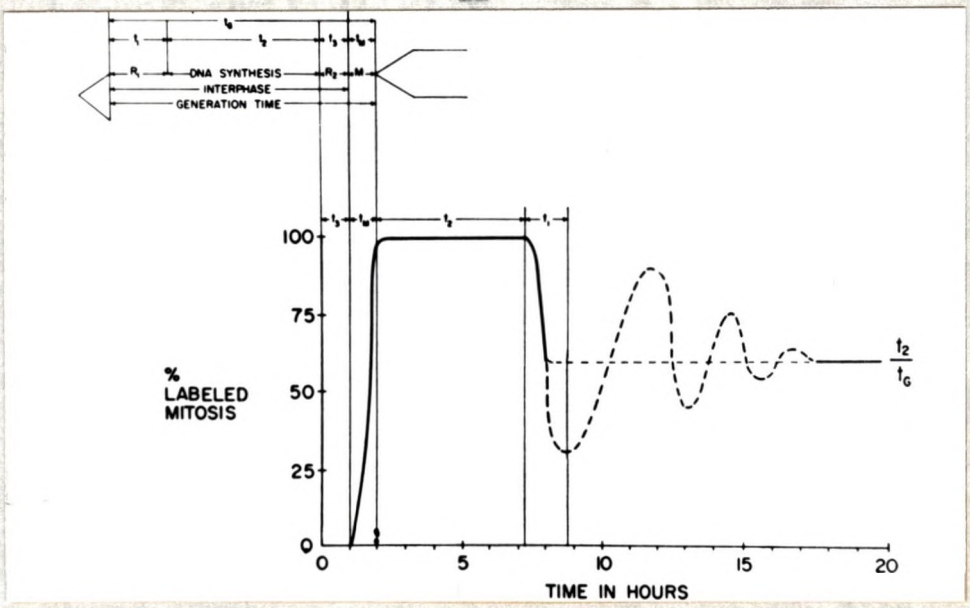


Text Figure I. The cell generative cycle. (Modified from Lajtha, Oliver and Ellis, Br. J. Cancer 8:367, 1954.).

The G_1 period has been referred to as the "pre-synthetic rest period". The length of this period is variable, especially in cell systems that divide upon demand, such as in the liver or periosteum (106). The second period

(T_S) is the time during which DNA synthesis occurs. Only cells that are in this period of generative cycle will take up the H^3TDR label. If a cell population is homogenous and completely asynchronous, then the percentage of labeled cells present (labeling index = I_L) a short time after the brief labeling period measures the percentage of the total generation time (T_G) during which the cells can synthesize DNA (T_S) (111). That is, if the labeling index (I_L) is expressed as the ratio of the labeled cells (N_2) to the total cells (N), and this ratio approximately measures the percentage of total generation time (G_T) occupied by the period of DNA synthesis (T_S), then $I_L = N_2/N = T_S/T_G$, and if the DNA synthesis time is known the average generation time can be computed directly (106). The G_2 period is called the "post-synthetic rest period" and is generally found to have a minimum time of 1 hour and a maximum time of 3 to 4 hours (106). The fourth period is the period of actual cell division (M).

The values for the periods of the generative cycle have been arrived at from the consideration of the percentage of labeled mitotic figures present in a cell population at various times. The H^3TDR is generally simultaneously injected into a number of experimental subjects which are then killed serially and the cell population under investigation is examined for the percentage of labeled cells that are mitotic figures (Text Figure 2). The time from injection of the isotope until the appearance of the first labeled mitotic figure gives the minimum time for completion of DNA synthesis and the post-synthetic rest period (T_3). According to the terminology of Cronkite et al. (103). If reference is made to Text Figure 1, this period of time approximates the minimum for G2



Text Figure 2. A graph of percent labeled mitotic figures against time demonstrating the length of the periods of the cell generative cycle. (Taken from Cronkite et al., Lab. Invest. 8:263, 1959.)

(106). The duration of time for the percentage of labeled mitotic figures to increase to approximately 100 percent represents the sum of distribution of T_3 and mitotic time (M_T). In reference to Figure 1, it represents the range in time for minimum to maximum G_2 (106). The length of time that labeled mitotic figures remain at 100 percent estimates DNA synthesis time (T_2 in Text Figure 2, Text Figure 1). As the cells that were in the pre-synthetic rest period enter mitosis the percentage labeling of mitotic figures is diluted by non-labeled mitotic figures. The curve will rise again as the labeled daughter cells enter the next mitosis, but the labeling intensity will be decreased by half with each mitosis (103).

Once the normal span of time has been established for each period of the generative cycle in a given cell population, these methods of evaluation may be utilized to study the effects of various experimental proceedures on each time period of the generative cycle. The index of labeling ($I_L = N_2/N$) has been especially useful in studying the changes in the fractions of cells that are synthesizing DNA under experimental

influence. In this investigation, the effect of a lathyrogen on the labeling index of various cell populations will be studied in an attempt to determine if lathyrogenic action initially influences normal mitotic activity.

CHAPTER II

MATERIALS AND METHODS

I. General Materials and Methods

Fifty-eight weanling female albino rats of the Holtzman strain, with an average weight of 49 grams at the start of the experiments, were used in this study. All animals were maintained on a daily regimen of Purina Laboratory Chow and fresh tap water. Lathyrism was induced in the experimental animals on their 21st or 22nd day of life by giving daily intraperitoneal injections containing 30 milligrams of beta-aminopropionitrile (BAPN) in 1 milliliter of distilled water. Cell populations synthesizing DNA in experimental and control animals were labeled by giving intraperitoneal injections of tritiated thymidine (H^3TDR, sp. Act. 1.9 c/mmole, Schwarz Bioresearch Inc.) at dosage of 0.5 microcurie per gram of body weight. Some control animals were also injected with a sodium chloride (NaCl) solution which was isotonic to the BAPN solution that was injected into the experimental rats. All animals were sacrificed by injecting an overdose of sodium pentobarbital. Three groups of animals were used.

Group I consisted of 10 experimental and 10 control animals. This group was designed to test the effect of BAPN upon the labeling indicies of cell populations that were synthesizing DNA at the time of initial BAPN injection. Consequently, BAPN and H^3TDR were injected simultaneously into the experimental animals. Experimental animals were sacrificed at

1 hour, 4 hours, 8 hours, 12 hours, 16 hours and 20 hours after the injections. Control animals were injected with H^3TDR and sacrificed at the same time periods.

Group II consisted of 16 experimental and 11 control animals. This group was designed to test the effect of BAPN on the labeling indicies of various cell populations at various time periods after the initial injection of BAPN. Each experimental animal received 1 injection of BAPN daily. Experimental animals were sacrificed at 4 hours, 8 hours, 12 hours, 16 hours, 20 hours , 24 hours, 2 days, 4 days and 7 days after the initial injection of BAPN. Each experimental animal was injected with H^3TDR 2 hours prior to sacrifice. Three control animals were injected intraperitoneally with 1 milliliter of NaCl solution and then given the intraperitoneal injection of H^3TDR 2 hours prior to sacrifice at 4 hours, 8 hours and 12 hours. The remaining control animals were injected only with H^3TDR two hours prior to sacrifice. At least 1 control animal was killed at each time period, with the exception of the 4 day time period. Six animals of this group were found to have a pneumonitis of unknown origin and were replaced by healthy animals. However, the labeling indicies of the various cell populations studied in the sick animals revealed some interesting differences when they were compared to the labeling indicies obtained from animals without the pneumonitis. Consequently, these animals were used separately to point out the effect of a disease process upon the labeling indicies of the cell populations studied. Evidence of an inflammatory reaction was found in microscopic sections taken from the

lungs of these animals (Figures 1 and 2). The lungs of the remaining animals were found to be clear of inflammation. (Figure 3).

Group III consisted of 9 experimental and 2 control animals. This group was designed primarily to trace the origin and migration of the various cell types that appear in the exostosis that develops at the site of adductor longus-pectineus insertion in lathyric rats. A large number of control animals was not considered necessary for this group because, once exostosis formation begins, there is no comparable situation in the periosteum at the site of insertion in normal rats. In addition, only a limited supply of H^3TDR could be obtained. Group III was subdivided into Group IIIa and IIIb for descriptive purposes. The 5 experimental animals of Group IIIa were injected with H^3TDR 24 hours after the initial injection of BAPN and sacrificed at 2 days, 4 days and 7 days after the initial injection of BAPN. Two control animals were also injected with H^3TDR and sacrificed with the 2-day and 7-day experimental animals. Group IIIb consisted of 4 experimental animals that were injected with H^3TDR after the animals had received 4 daily injections of BAPN and sacrificed at 7 days, 9 days and 12 days after the initial injection of BAPN. The 12-day experimental animal received an additional injection of H^3TDR 2 hours prior to sacrifice. Cells taking up the H^3TDR label that was injected 2 hours prior to sacrifice were demonstrated by exposing the emulsion for 30 days while cells retaining some of the H^3TDR label that was injected on day 4 were demonstrated by exposing the emulsion for longer periods of time.

After sacrifice, tissue was taken from the middle third of each femur containing the periosteum at the site of adductor longus-pectineus insertion, the proximal tibial epiphyseal plate, the adrenal glands and from the lungs. The lung sections were used to determine if the rats had any microscopic signs of respiratory disease.

All tissue was fixed in Bouin-Hollande fixative for 3 days (112). Bone material was decalcified in a 5 percent solution of ethylenediaminetetraacetic acid (EDTA) at pH 7. Paraffin embedded sections were cut at 6 micra and mounted on chemically clean glass slides. A routine hematoxylin and eosin proceedure was performed on samples of all tissue. Sections to be used for autoradiography were either stained with the periodic acid-Schiff reaction before dip-coating (112) with Kodak NTB2 nuclear emulsion, and counter-stained with Harris hematoxylin after the emulsion was developed, or stained with hematoxylin alone. The emulsion was exposed from 30 to 50 days in a cold, dry atmosphere. After exposure, the emulsion was developed according to the following proceedure; (1) 2 minutes in undiluted Kodak Dektol at 17°C., (2) a brief water rinse (17°C) , (3) 15 minutes in "hypo" fixer at 17°C., (4) washed in running tap water for at least 1 hour, (5) dehydrated in an ascending alcohol series, (6) passed through 2 changes of xylene, and (7) mounted in permount and coverslipped.

II. METHODS OF ANALYSIS

A. The periosteum at the Site of Adductor Longus-Pectineus Insertion.

Yeager and Hamre (61) have reported that the long fibers of the pectineus muscle and the tendon of the adductor longus muscle form a

common functional insertion at a point slightly distal to the middle of the femur shaft. The periosteum at this site is greatly thickened, but gradually thins out along the circumference of the bone to become continuous with the thin general periosteum. Histologically, the periosteum at this site is composed of a thin outer fibrous layer and an inner thickened osteogenic layer (Figure 4). The osteogenic layer of periosteum is the layer in which exostoses develop in lathyric rats. The majority of the cell population of the osteogenic layer of periosteum is composed of fibroblast-like periosteal cells. However, endothelial cells, peri-capillary cells, preosteoblasts and osteoblasts are also present in normal rats (Figure 5). Since it was the principle interest of this study to determine when the cells were affected by BAPN, the pre-osteoblasts and periosteal cells were counted as one cell population in 5 sections from each insertion. The sections were chosen so that 2 sections distal to the approximate center of the insertion, 1 section at the approximate center of the insertion, and 2 sections proximal to the approximate center were examined (Figure 21). The total population of periosteal cells and preosteoblasts was counted in 2 to 3 high power fields (43 x) from each section. After the total cell population present in each field was determined, the field was re-examined and the periosteal cells and preosteoblasts with 4 or more silver grains over their nuclei were counted. This proceedure was observed in all control animals and in the experimental animals up until 4 days after the initial BAPN injection. At 4 days the osteogenic layer of periosteum at the insertion site was noticeably enlarged and an increased amount of intercellular material was noted in the deeper areas of the periosteum (Figure 6). It was

noted that the largest number of labeled cells were located in an area between the fibrous layer of periosteum and the area of increased intercellular material. Thus, an arbitrary line was drawn above the area of increased intercellular material and only the cells located above the line were counted (Figure 7). This method of counting was also followed in the 7-day developing exostosis (Figure 8).

B. The Proximal Tibial Epiphyseal Plate.

After fixation, the proximal portions of the tibiae were bisected longitudinally as near to the midline as possible. Only sections from near the midline were utilized in determining the labeling indicies of the epiphyseal plates. The cell counts were carried out in the following manner.

With the aid of an ocular grid, an area .31 millimeters in length and .13 millimeters in width was measured before each high power field was counted. This area generally extended from the top of the zone of proliferating cartilage cells to approximately the upper fourth of the zone of maturing cartilage cells (Figure 9). At least 2 sections of epiphyseal plate from each animal were examined by counting the total cartilage cell population and labeled cartilage cell population in 4 to 6 high power fields across each epiphyseal plate.

C. The Adrenal Gland

The rat adrenal cortex consisted of three distinct zones (Figure 10). (1) The zona glomerulosa, which was located immediately below the capsule of the gland. The parenchymal cells of this zone are organized into loops and arches. A transition zone was located immediately below the

glomerulosa. Some authors (113) consider the transition zone to be within the glomerulosa . (2) The zona fasciculata was the thickest of the three zones. The parenchymal cells of this zone contain round, vesicular nuclei and are arranged in radial cords bordered by capillaries. (3) The zona reticularis was located immediately adjacent to the adrenal medulla and was characterized by an irregularly arranged network of cells with dark staining, ofter pyknotic nuclei.

Labeling of adrenal cortical cells with H^3TDR has shown that the zona glomerulosa (plus the transition zone) is the zone in which the majority of cell proliferation occurs (113,114). From this zone the cells apparently migrate centripetally (113,115).

The parenchymal cells of the zona glomerulosa, including the transition zone, were counted as one cell population. Six to 8 random high power fields were counted for each adrenal. In addition, the upper .1 millimeter of the zona fasciculata was counted in the adrenal glands of the Group I animals in an attempt to determine if an increased centripetal migration could be observed during the time period studied (Figure 10).

The labeling index (I_L) of each cell population investigated was determined by the equation $I_L = N_2/N \times 100$, where $N_2 =$ number of labeled cells counted, and $N =$ total number of cells counted.

CHAPTER III

RESULTS

I. GROSS OBSERVATIONS AND AUTOPSY EXAMINATION

Observation of control and experimental rats before they were sacrificed indicated that some of the animals were experiencing respiratory distress which was manifested as sniffles with an occasional sharply exhaled breath of air. At autopsy, the external surfaces of the lungs of 5 experimental and 1 control rat of Group II were found to be covered with grey-white spots. The most severely spotted lungs were found in the 4 and 7-day lathyric rats. There were no other unusual findings observed at autopsy in these animals. As mentioned in Chapter II, these animals were replaced by healthy rats, but the results obtained from the sick animals were used to point out the effect of a disease process upon the labeling indicies of the cell populations studied.

The remaining control and experimental animals appeared to be healthy. None of the experimental animals showed any grossly observable effects of BAPN toxicity during the course of the experiment. Autopsy examination showed the periosteum at the site of the adductor longus-pectineus insertion to be enlarged in the 4 and 7-day lathyric rats. All other organs appeared normal at autopsy.

II. THE PERIOSTEUM AT THE SITE OF ADDUCTOR LONGUS-PECTINEUS INSERTION

A. Normal Morphology (Hematoxylin and Eosin)

The periosteum in this area consisted of an outer fibrous layer and an inner osteogenic layer.

The fibrous layer of periosteum was only slightly thicker than the fibrous layer of the general periosteum. A direct continuity of the dense collagen bundles in the fibrous layer at the site of insertion with the collagen bundles of the fibrous general periosteum was noted. Spindle-shaped fibroblasts with elongated nuclei were located between the bundles of collagen fibers (Figure 4).

The osteogenic layer of periosteum at this site was greatly increased in thickness when compared to the osteogenic layer of general periosteum (Figure 4). Three principle cell types were present in this layer.

A row of osteoblasts lined the cortical bone of the femur (Figure 5). These osteoblasts exhibited the normal osteoblast morphology with a very basophilic cytoplasm, spherical nucleus and 1 or 2 prominent nucleoli. A juxta-nuclear vacuole was noted in the cytoplasm of some of the osteoblasts. Most of the osteoblasts appeared to be very active. Their morphology was similar to that of the osteoblasts located on the periphery of the newly formed bone spicules in the developing exostoses of the lathyric animals.

Immediately peripheral to the row of periosteal osteoblasts was an irregular layer of basophilic, irregularly shaped cells that were considered to be preosteoblasts (Figure 5). The term "preosteoblast" was used to designate these cells because they were quite similar to osteoblasts, but had not yet acquired the functional differentiation or

topographical relations of a mature osteoblast.

The area extending from the area occupied by the preosteoblasts to the fibrous layer of periosteum was populated by fibroblast-like periosteal cells. The reasons for distinguishing these cells from fibroblasts were given in a previous paper (87). The cytoplasm of the periosteal cells was slightly basophilic and elongated. The periosteal cell nuclei were fusiform to oval in shape and many of them appeared quite vasicular. However, smaller, darker staining nuclei were also observed in these cells. One or 2 nucleoli could usually be observed in the nuclei of the periosteal cells. The periosteal cells were generally oriented so that one pole of the cell pointed toward the femur while the other pole of the cell pointed toward the tendon of insertion (Figure 5). This orientation was due to the fact that the collagen fibers in the osteogenic layer of periosteum at this site run from the tendon of insertion toward the cortical bone of the femur, and the periosteal cells were located between the collagen fibers.

B. Morphology after BAPN Treatment (Hematoxylin and Eosin)

The fibrous layer of periosteum showed no morphological changes during exostosis development. However, the fibrous layer of periosteum did contain the bulging exostoses that developed and therefore, must have undergone some alteration during exostosis development.

Yeager and Hamre (61) have adequately described the histology of the exostosis that develops in the periosteum at the site of adductor longus-pectineus insertion in adult lathyric rats. They divided exostosis

formation into two periods; (1) a proliferative period during which there was a day by day increase in the number of mitotic figures per cubic millimeter of tissue, and which began after one day on a Lathyrus diet and continued through day 6, and (2) an osteogenic period of exostosis formation that began with bone formation in the osteogenic periosteum in the 7 or 8-day adult lathyric rat. Ollerich (87) has further pointed out that one of the initial changes in the periosteal cells at the site of insertion was a decrease in ribonucleic acid (RNA) staining intensity accompanied by morphological changes that suggested dedifferentiation of the periosteal cells. Two days after starting BAPN treatment the nuclei of the periosteal cells were larger, more vesicular and contained large, prominent nucleoli. Some variation from this pattern was noted in the exostoses developing in the immature rats used in this study.

It has previously been mentioned that the osteogenic layer of periosteum at the site of insertion in control animals contained many periosteal cells with rounded, vesicular nuclei. There was no visually apparent increase in the size or number of these nuclei in the 2-day lathyric rats used in this study (Figure 11). In addition, an increase in intercellular material and some bone formation was noted in the deeper areas of the osteogenic periosteum in the 2-day lathyric rats (Figure 11). However, an increase in the size of the periosteal cell nuclei and nucleoli was noted in the 4-day developing exostoses (Figure 12). The osteogenic periosteum at the site of insertion was noticeably enlarged in the 4-day lathyric rats, and a definite increase in intercellular material, as well as some new bone formation was evident (Figure 13). Thus, the three

zones originally described by Yeager and Gubler (86) in 7-day developing exostoses were essentially present in the osteogenic layer of periosteum at the site of insertion in the immature 4-day lathyric rats used in this study. However, the zones were not as definite as in the osteogenic layer of periosteum of the 7-day, 9-day and 12-day lathyric rats (Figure 14). These three zones were; (1) a proliferative zone located just subjacent to the fibrous layer of periosteum and composed of many periosteal cells with large, vesicular nuclei, (2) a zone of intercellular material composed of much intercellular material and a sparse cell population, and (3) a zone of intramembranous bone formation composed of preosteoblasts, osteoblasts and newly formed bone.

The periosteum at the site of insertion in the 4 and 7-day lathyric rats with pneumonitis showed only slight lathyric changes (Figure 15). An increase in intercellular material was noted in the osteogenic layer of periosteum at the site of insertion in the 7-day lathyric rat with pneumonitis, but a definite proliferative zone and a zone of intramembranous bone formation were lacking.

C. Normal Distribution of H^3 TDR Labeled Cells.

Autoradiographs of sections taken from the periosteum at the site of insertion in control rats from all three groups demonstrated that the H^3TDR-label had been taken up by some of the fibroblast nuclei present in the fibrous layer. There was no apparent localization of labeled cells and label distribution appeared to be random, (Figure 16).

Autoradiographs of sections taken from the thickest portion of the periosteum near the center of insertion in control animals of Group I and Group II showed that a zone of labeled cells was located directly below the fibrous layer of periosteum. In the control animals of Group II, that were sacrificed 2 hours after H^3TDR injection, this outer zone generally extended entirely across the superior portion of the osteogenic layer of periosteum from one periphery of the insertion to the other (Figure 16). The number of labeled cells decreased quite abruptly beneath this zone and the area in the center of the osteogenic layer of periosteum did not contain many labeled cells (Figure 16). As the cortical bone of the femur was approached a rather indefinite and irregular zone of labeled cells was noted (Figure 16). The labeled cells in this zone were predominately preosteoblasts, but an occasional labeled osteoblast was noted (Figure 17). The inner zone of labeled cells was not as evident as the outer zone of labeled cells. Generally, the inner zone was incomplete and only rarely could be seen to extend across the inferior aspect of the osteogenic layer of periosteum without interruption. However, examination of several sections from this area gave the impression that the H^3TDR-label was being taken up by cells in this zone across the entire extent of the insertion.

The control animals of Group I were sacrificed at progressively longer periods after H^3TDR injection and all three zones showed an obvious increase in the number of labeled cells with time. This increase was most obvious in the outer zone, but the inner zone and the central zone also showed an increased number of cells with silver grains over their

nuclei. There also appeared to be more osteoblasts labeled per section at the later time periods. In the 16 and 20-hour control animals of this group, it was obvious that the outer zone had progressively increased in width and had encroached somewhat on the central zone (Figure 18). In some sections from these animals the outer and inner zones of labeled cells could be seen to merge at the periphery of the insertion, giving rise to an area in which a high percentage of the cells exhibited silver grains over their nuclei (Figure 19). In addition, autoradiographs of sections taken both distal and proximal to the center of insertion, where the thickened osteogenic periosteum progressively narrowed to join the osteogenic layer of general periosteum, showed that the inner and outer zone of labeled cells converged until the sparsely labeled central zone could no longer be easily noted. Thus, the osteogenic layer of periosteum appeared to be more uniformly labeled (Figure 20). A summary of the distribution of labeled cells in the osteogenic layer of periosteum at the site on insertion in a control animal sacrificed 16 hours after H^3TDR injection is given in Figure 21.

Since the 2-day control animal of Group IIIa was sacrificed 24 hours after injection of H^3TDR, the distribution of labeled cells in the osteogenic periosteum at the site of insertion was essentially the same as that just described for the 20-hour Group I control. The Group IIIa 7-day control rat was sacrificed 6 days after the H^3TDR injection. Very few cells with silver grains over their nuclei were noted in the osteogenic periosteum of this animal even after long term exposure of the emulsion. However, some labeled osteoblasts and osteocytes were noted (Figure 22).

D. Distribution of Labeled Cells after BAPN Treatment

Autoradiographs of sections of periosteum from the site of insertion indicated no visually observable changes in the distribution of labeled cells in the fibrous layer after BAPN treatment.

The distribution of labeled cells in the osteogenic layer of periosteum at the site of insertion in the lathyric rats of Group I was similar to that in Group I control animals with the majority of labeled cells located in an inner and outer zone (Figure 23).

Autoradiographs of sections from the developing exostoses of Group II lathyric rats showed that the distribution of labeled cells was also similar to the distribution in Group II control rats up to 12 hours after the initial injection of BAPN. At this time some sections from near the center of the insertion contained numerous labeled cells in all three zones (Figure 24). The random distribution of labeled cells in the osteogenic layer of periosteum was progressively more evident and in the 2-day lathyric rat the osteogenic layer of periosteum appeared to have many labeled cells in all three zones throughout the entire extent of the insertion (Figure 25). Four days after the initial injection of BAPN the majority of labeled cells were located in a fairly definite proliferative zone directly beneath the fibrous layer of periosteum and outside the zone of intercellular material (Figure 26). However, sections taken from near the center of insertion or proximal to the center of insertion contained more labeled cells in the proliferative zone than did sections taken distal to the center of insertion. This was also true in the 7-day and 12-day developing exostoses (Figure 27 and 28). However, autoradiographs of sections taken from near the center of insertion or proximal

to the center of insertion in the 7 and 12-day lathyric rats showed that a large number of the labeled cells were concentrated on the medial, or pectineus side of the insertion (Figure 29). This was especially evident in the 12-day developing exostosis (Figure 28).

Labeled cells were also present in the zone of intercellular material and zone of intramembranous bone formation in the 4, 7 and 12-day developing exostoses (Figure 30). The majority of labeled cells in the zone of intercellular material were located near the outer edge of the zone (Figure 30). The number of labeled cells appeared to decrease from out inward. However, labeled preosteoblasts and osteoblasts were present in the zone of intramembranous bone formation (Figure 31).

Autoradiographs of sections taken from the osteogenic layer of periosteum in the 2-day lathyric rats of Group IIIa showed that the distribution of label was more random in most areas of the insertion than it was in the Group IIIa control (Figure 32). However, sections taken from near the center of insertion generally contained a high number of labeled cells at the periphery of the insertion. Autoradiographs of sections taken from a Group IIIa 4-day developing exostosis demonstrated a light accumulation of silver grains over the nuclei of some cells in all three zones. An occasional heavily labeled cell was noted in the proliferative zones. More cells were noted in the zone of intercellular material that had a relatively heavy accumulation of silver grains over their nuclei (Figure 33). In addition, several preosteoblasts, some osteoblasts and an occasional labeled osteocyte were noted in the zone of intramembranous bone formation (Figure 33). The Group IIIa 7-day developing exostoses

contained labeled cells primarily in the zone of intercellular material and in the zone of intramembranous bone formation. Preosteoblasts, osteoblasts and osteocytes were found to be labeled in the zone of intramembranous bone formation (Figure 34). It appeared that there were more silver grains over the nuclei of these cells than over the nuclei of the labeled cells in the zone of intercellular material (Figure 34). Since no grain counts were made, this finding was not definite and may have been due to a difference in nuclear size.

Before discussing the distribution of labeled cells in the developing exostoses from 7, 9 and 12-day lathyric rats of Group IIIb, it should be recalled that the proliferative zone of the Group II 4-day developing exostosis contained the majority of labeled cells (Figure 26). The Group IIIb experimental animals were injected with H^3TDR 4 days after the initial injection of BAPN.

The Group IIIb 7-day developing exostosis showed what appeared to be a random distribution of labeled cells in all zones (Figures 34). The proliferative zone of the 9-day developing exostosis contained some lightly labeled cells, but rarely any distinctly labeled cells (Figure 35). The zone of intercellular material contained many cells with a light accumulation of silver grains over their nuclei and some cells with relatively heavy accumulations of silver grains over their nuclei, while the zone of intramembranous bone formation contained some relatively heavy accumulations of silver grains over the nuclei of preosteoblasts, osteoblasts, and osteocytes (Figure 36). Very little evidence of the

H^3TDR-label that had been injected on day 4 was found in the 12-day developing exostosis. An occasional labeled osteocyte was noted.

Thus, a comparison of the distribution of labeled cells in the osteogenic layer of periosteum at the site of adductor longus-pectineus showed that the majority of labeled cells were distributed in an outer zone of labeled periosteal cells and an inner zone of labeled pre-osteoblasts in Group I experimental and control animals and in the Group II control and experimental animals up to 12 hours after the injection of BAPN. At that time more labeled cells were evident in the normally sparsely labeled central zone. The osteogenic layer of periosteum from the Group II 2-day lathyric rat contained labeled cells randomly distributed in all zones. The majority of labeled cells in the developing exostoses of the Group II 4-day and 7-day lathyric rats and in the specially treated 12-day lathyric rat were distributed in a definite proliferative zone directly beneath the fibrous layer of periosteum and outside the zone of intercellular material. More labeled cells were present in the proliferative zone of these animals in sections taken from near the center of insertion or proximal to the center of insertion. Autoradiographs of the developing exostoses from Group IIIa and IIIb lathyric rats showed that cells with heavy accumulations of silver grains over their nuclei disappeared first from the proliferative zone and then from the zone of intercellular material. Finally, only an occasional labeled osteoblast and osteocyte in the zone of intramembranous bone formation showed relatively high concentrations of silver grains over their nuclei.

E. The Labeling Indicies of Group I. (Osteogenic Layer of Periosteum)

The values obtained for the percent of cells labeled in the osteogenic layer of periosteum of control and experimental animal of Group I are given in Table 1.

TABLE 1. - Percent labeled (I_L) cell population in the osteogenic layer of periosteum at the site of adductor longus-pectineus insertion of BAPN-treated and the control rats of Group I.

Time sacrificed after injection (in hours)		Number of Animals		Percent of Labeling (I_L)	
BAPN	H^3TDR	Lathyric	Control[a]	Lathyric	Control
1	1	1	1	5.6	4.9
4	4	2	2	8.4[b]	8.4[b]
8	8	2	2	10.6	9.1
12	12	2	2	16.4	9.3
16	16	2	2	18.4	15.7
20	20	1	1	15.5	19.8

[a] Control animals received only H^3TDR injections.

[b] Where more than 1 animal was used, the value given is an average value.

An average of 2800 cells was counted per animal.

Calculation of the percent of cells labeled in the osteogenic layer of periosteum in BAPN-treated and control rats of Group I showed that the values obtained for the BAPN-treated rats began to increase above control values 8 hours after the injections had been given (Table 1). At this time the average labeling index for 2 experimental animals, was 1.5 percent higher than the average labeling index for 2 control animals.

At 12 hours post-injection, the average value for 2 experimental animals was 7.1 percent higher than that obtained for the 2 control animals. There was no overlap of values at this time, but one control animal exhibited a very low labeling index and consequently depressed the average value obtained for the control animals. The difference between the average labeling indicies of experimental and control animals, 16 hours after they received their injections was 2.7 percent. The experimental values ranged from 17.7 percent to 19.0 percent, while the control values ranged from 15.4 percent to 16.0 percent. Twenty hours after the injections were given, the value for the labeling index of the experimental animal was lower than that of the control animal.

F. The Labeling Indicies of Group II (Osteogenic Layer of Periosteum)

The initial effect of BAPN on the percent of cells labeled in the osteogenic layer of periosteum of Group II animals appeared to be a depression in the number of cells that were able to take up the H^3TDR label during the early time periods following BAPN injection (Table 2). The decrease in the percent of cells labeled was first noted 4 hours after the injection of BAPN and was more evident 8 hours after the injection of BAPN. At this time, the average experimental labeling index was 4.2 percent, with a range of 3.9 percent to 4.5 percent. These values were lower than either the value obtained from the 8-hour saline control or the control that was injected only with H^3TDR. In addition, the 8-hour experimental values were lower than any control values obtained over the first 24 hour period. The average labeling index of 3 experimental animals

TABLE 2. - Percent labeled (I_L) cell population in the osteogenic layer of periosteum at the site of adductor longus-pectineus insertion of BAPN-treated and control rats of Group II.

Time sacrificed after injection		Number of animals		Percent of labeling (I_L)	
BAPN	H^3TDR	Lathyric	Control	Lathyric	Control
4 hrs	2 hrs	2	1 1	4.9[a]	7.5 6.3[b]
8 hrs	2 hrs	2	1 1	4.2	8.2 8.8[b]
12 hrs	2 hrs	3	1 1	12.5	8.4 7.6[b]
16 hrs	2 hrs	2	1	11.1	8.4
20 hrs	2 hrs	2	1	12.5	5.2
24 hrs	2 hrs	2	1	14.3	8.8
2 days	2 hrs	1	1	15.2	5.9
4 days	2 hrs	1	-	9.8	-
7 days	2 hrs	1	1	17.4	1.6

[a] Where more than 1 animal was used, the value given is an average value.

[b] This designation indicates the values obtained from control animals that were injected with NaCl solution at the same time the experimental animals were injected with BAPN solution. The remaining control animals received only H^3TDR injections.

increased abruptly to 12.5 percent 12 hours after the injection of BAPN. The 3 experimental values were 10.6 percent, 13.4 percent and 13.5 percent.

Each of these values was higher than any control value obtained during the entire time period studied. The percent of cells labeled in the osteogenic layer of periosteum in experimental animals were consistantly higher than the percent of cells labeled in the osteogenic layer of periosteum in experimental animals throughout the remainder of the time period studied.

The labeling indicies of the Group II control animals showed some variation during the first 24 hours. There was no consistant difference between the NaCl injected control animals and the control animals that were injected only with H^3TDR. The 4-day control animal of this group showed evidence of respiratory disease.

II. THE PROXIMAL EPIPHYSEAL PLATE

A. Normal Morphology (Hematoxylin and Eosin)

The morphology of the epiphyseal plates of the control rats used in this study was similar to that described in textbooks of histology. The terminology in the description given below was taken from Ham and Leeson (116).

The epiphyseal plates of control rats could be microscopically divided into 4 zones.

The zone of resting cartilage was the most proximal zone of the epiphyseal plate. This zone was rather thin and contained randomly scattered chondrocytes throughout the cartilage matrix (Figure 9). The superior edge of this zone was generally in contact with a thin layer of epiphyseal bone.

Immediately below the preceeding zone was the zone of proliferating cartilage cells (Figure 9). The cartilage cells of this zone were usually very thin and arranged in irregular vertical rows. The vertical rows of cartilage cells were separated from each other by layers of cartilage matrix. Many mitotic figures were noted and often 2 cartilage cells could be seen occupying the same lacuna.

The zone of maturing cartilage cells was composed of vertical rows of cartilage cells that increased in size from above downward (Figure 9). The nuclei of these cells also became larger and more vesicular and toward the bottom of the zone some degenerated cells with no visable nuclei could be seen. As the maturing cartilage cells hypertrophied, the vertical rows became more disorganized and the layer of cartilage matrix between the rows became thinner.

The zone of calcifying cartilage was relatively thin and ended directly upon the developing bone trabeculae of the metaphysis. The cartilage cells were generally disintegrated and many lacunae were completely empty (Figure 9).

B. Morphology after BAPN Treatment (Hematoxylin and Eosin)

The only morphological change noted in the proximal tibial epiphyseal plates of lathyric rats was a disorganization of the orderly arrangement of cellular components in the various zones. This change was first noted in some of the 7-day lathyric rats and was somewhat more evident in the epiphyseal plate of the 9-day lathyric rat (Figure 38).

C. Normal Distribution of H^3TDR-Labeled Cartilage Cells.

Autoradiographs of sections from the proximal tibial epiphyseal plates of Group II control animals that were sacrificed 2 hours after H^3TDR injection, demonstrated that the labeled cartilage cells were confined to the area previously described as the zone of proliferating cartilage cells (Figure 39). Autoradiographs of sections from the epiphyseal plates of control animals of Group I sacrificed at progressively longer periods after H^3TDR injection showed that there was a downward displacement of some of the labeled cells. Cells with silver grains over their nuclei were present in the upper portion of the zone of maturing cartilage cells 16 hours after injection of the H^3TDR label (Figure 40). The downward displacement of labeled cells was more evident in animals sacrificed at later time periods. Six days after the injection of H^3TDR label some light concentrations of silver grains could be found over the nuclei of cartilage cells throughout the zone of proliferating cartilage cells and the zone of maturing cartilage cells.

D. Distribution of H^3TDR-Labeled Cartilage Cells after BAPN Treatment.

There was no visually recognizable change in the distribution of labeled cartilage cells in the proximal tibial epiphyseal plates of the lathyric rats in any of the experimental groups.

E. The Labeling Indicies of Group I (Epiphyseal Plates)

The values obtained for the percent of cells labeled in the proximal tibial epiphyseal plates of control and experimental animals of Group I are given in Table 3.

Calculation of the percent of cells labeled in the epiphyseal plates of control and BAPN-treated rats indicated that there were more labeled cartilage cells present in the lathyric epiphyseal plate at one hour after the injections were given. However, 4 hours after the

TABLE 3. - Percent labeled (I_L) cell population in the proximal tibial epiphyseal plates of BAPN-treated and control rats of Group I.

Time Sacrificed after injection (in hours)		Number of animals		Percent of Labeling (I_L)	
BAPN	H^3TDR	Lathyric	Control[a]	Lathyric	Control
1	1	1	1	8.7	7.6
4	4	2	2	12.4[b]	14.2[b]
8	8	2	2	19.9	14.5
12	12	2	2	19.9	17.6
16	16	2	2	20.9	15.5
20	20	1	1	10.7	14.2

[a] Control animals received only H^3TDR injections.

[b] Where more than one animal was used, the value given is an average value.

An average of 1187 cells was counted per animal.

injections were given the average labeling index for 2 control rats was 1.8 percent higher than the average labeling index of 2 experimental animals. The values obtained at the first two time periods were considered to be normal variation, since the next 3 successive time periods showed that the average labeling index of the BAPN-treated rats was consistantly higher than the average labeling index of the control rats (Table 3). The average labeling index for 2 experimental animals was 5.4 percent higher

at 8 hours post-injection, 2.3 percent higher at 12 hours post-injection and 5.4 percent higher at 16 hours post-injection when compared to the respective control values. The only overlap between experimental and control values was noted at 16 hours after the injections were given. The labeling index of the 20-hour lathyric rat was lower than the 20-hour control value.

B. The Labeling Indicies of Group II (Epiphyseal Plates)

The percent of cells labeled in the proximal tibial epiphyseal plates in BAPN-treated and control animals of Group II are given in Table 4.

The first difference between the percent of cells labeled in the proximal tibial epiphyseal plates of BAPN-treated and control animals was noted 8 hours after the injection of BAPN (Table 4). At this time the average value for two experimental animals was less than the value obtained for either of the 8-hour control animals. In addition, the average labeling index of the 8-hour lathyric epiphyseal plate was lower than any control value obtained throughout the entire time period investigated. By 12 hours post-BAPN injection the percent of cells labeled in the lathyric epiphyseal plates had returned to control values. The average experimental labeling index then gradually increased to become definitely higher than control values by 20 hours post-BAPN injection. The experimental labeling indicies remained higher than the control labeling indicies throughout the remainder of the time period studied.

No consistant difference was noted between the labeling indicies of the control rats that were injected with NaCl solution and the control

TABLE 4. - Percent labeled (I_L) cell population in the proximal tibial epiphyseal plate of BAPN-treated and control rats of Group II.

Time sacrificed after injection		Number of animals		Percent of labeling (I_L)	
BAPN	H^3TDR	Lathyric	Control	Lathyric	Control
4 hrs	2 hrs	2	1	9.6[a]	8.7 10.0[b]
8 hrs	2 hrs	2	1 1	6.4	7.9 9.8[b]
12 hrs	2 hrs	3	1 1	9.7	9.4 9.4[b]
16 hrs	2 hrs	2	1	10.7	9.3
20 hrs	2 hrs	2	1	14.6	10.4
24 hrs	2 hrs	2	1	14.2	10.0
2 days	2 hrs	1	1	15.7	11.5
4 days	2 hrs	1	-	16.0	--
7 days	2 hrs	1	1	15.3	11.0

[a] Where more than 1 animal was used, the value given is an average value.

[b] This designation indicates the values obtained from control animals that were injected with NaCl solution at the same time the experimental animals were injected with BAPN solution. The remaining control animals received only H^3TDR injections.

An average of 1165 cells was counted per animal.

rats that were injected only with H^3TDR. The percent of labeling values for the 2 and 7-day control epiphyseal plates were slightly higher than the control values obtained during the first 24 hour period.

III. THE ADRENAL CORTEX

A. Normal Morphology (Hematoxylin and Eosin)

The rat adrenal cortex was subdivided according to the work of Ford and Young (113).

The zona glomerulosa was the outermost layer of the cortex located immediately beneath the capsule of the gland. The parenchymal cells were grouped together in irregular groups and clusters (Figure 10). The nuclei of the glomerular parenchymal cells were usually round and stained rather darkly. The cytoplasm of these cells was lightly stained with eosin. An occasional darkly stained endothelial cell could be seen lining the capillaries between the clusters of parenchymal cells.

The transition zone was considered by Ford and Young (113) to be within the glomerulosa. Other authors have considered this zone to be the outermost part of the zona fasciculata (117). This zone was found to vary in density and thickness. The nuclei of cells in the transition zone were more irregular in shape and usually smaller than those in the glomerulosa or fasciculata. The cytoplasm of the transition zone cells was usually quite eosinophilic (Figure 10).

The zona fasciculata was the thickest layer of the rat adrenal cortex (Figure 10). The parenchymal cells of this zone were arranged in cords that were oriented at right angles to the surface of the adrenal. The fascicular cords were bordered by capillaries. The nuclei of the

parenchymal cells in this zone were large, round and lightly stained. Their cytoplasm stained very lightly with eosin and was highly vacuolated.

The zona reticularis abutted directly upon the adrenal medulla. The cells of this zone were arranged in an irregular, anastomosing manner. The nuclei of the cells in the reticularis were generally small and dark and the cytoplasm was very eosinophilic.

B. Morphology after BAPN Treatment (Hematoxylin and Eosin)

No changes in the morphology of the adrenal cortex were noted in any of the experimental animals.

C. Normal Distribution of H^3TDR-Labelled Cells.

Autoradiographs of sections taken from the adrenal glands of control animals of Groups I and II showed the majority of labeled cells to be present in the zona glomerulosa and transition zone (Figure 41). Some labeled cells were also found in the outermost part of the zona fasciculata and an occasional labeled cell was noted deep in the zona fasciculata (Figure 41). No labeled parenchymal cells were noted in the zona reticularis. Labeled endothelial cells were noted in all three zones.

D. Distribution of H^3TDR-labeled Cells After BAPN Treatment.

No change in the distribution of labeled cells in the adrenal cortex was noted in the experimental animals of Groups I and II.

E. The Labeling Indicies of Group I (Adrenal Cortex).

The percent of cells labeled in the adrenal cortex of BAPN-treated and control rats of Group I are given in Table 5.

TABLE 5. - Percent labeled (I_L) cell population in the adrenal cortex of BAPN-treated and control rats of Group I.

Time sacrificed after injection (in hours)		Number of animals		Percent of Labeling (I_L)			
BAPN	H^3TDR	Lathyric	Control[a]	Lathyric		Control	
				Glom.	Fas.	Glom.	Fas.[b]
1	1	1	1	4.6	1.3	5.3	1.4
4	4	2	2	4.1[c]	1.2	5.4	0.8
8	8	2	2	7.3	1.4	7.8	1.3
12	12	2	2	7.6	2.4	7.0	2.3
16	16	2	2	7.1	3.1	7.2	2.1
20	20	1	1	7.5	2.5	9.5	1.7

[a] Control animals received only H^3TDR injections.

[b] Only parenchymal cells in the outer.1 millimeter of the fasciculata were counted.

[c] Where more than one animal was used, the value given is an average value.

An average of 1100 cells was counted per glomerulosa, and an average of 1200 cells was counted per fasciculata.

No consistant trend was obvious when the labeling indicies of the cell populations investigated in the adrenal cortex of Group I BAPN-treated rats were compared to the labeling indicies of Group I control rats. The largest difference was found between the labeling indicies of experimental and control rats that were sacrificed 20 hours after they were injected (Table 4). At this time the control value was 2 percent higher than the experimental value. However, the labeling indicies of the cell

populations investigated in the osteogenic layer of periosteum at the site of insertion and in the proximal epiphyseal plates of this animal were also larger than the 20-hour lathyric values. Therefore, this difference was felt to be due to some factor other than the influence of BAPN. On the other hand, at 4 hours the average labeling index for 2 experimental animals was 1.3 percent lower than the respective control value. The difference between experimental and control values at this time period was felt to be due to normal variation between animals because no consistant trend was established. The results obtained for the labeling indicies of the outer portion of the zona fasciculata were also inconclusive. There was an overlap of experiment and control values at all time periods in which 2 experimental and 2 control animals were used. In contrast to the other cell populations studied, the labeling index of the outer portion of the zona fasciculata was larger in the 20-hour lathyric rat than in the control rat at that time.

F. The Labeling Indicies of Group II (Zona Glomerulosa and Transition Zone).

The values obtained for the labeling indicies of the cell population in the zona glomerulosa (including transition zone) of BAPN-treated and control rats of Group II are given in Table 6.

The values obtained for the percent of cells labeled in the zona glomerulosa (plus transition zone) of BAPN-treated rats of Group II were less than the values obtained for control animals at all time periods except at 20 hours post-BAPN injection (Table 6). The average labeling index of the 20-hour lathyric rats was 1.4 percent higher than the value obtained for the 20-hour control animal. The 20-hour experimental value

TABLE 6- Percent labeled (I_L) cell population in the zone glomerulosa plus transition zone of the adrenal cortex of BAPN-treated and control rats of Group II

Time Sacrificed after injection		Number of animals		Percent of labeling (I_L)	
BAPN	H^3TDR	Lathyric	Control	Lathyric	Control
4 hrs	2 hrs	2	1 1	4.7[a]	5.4 4.9[b]
8 hrs	2 hrs	2	1 1	3.5	4.7 4.6[b]
12 hrs	2 hrs	3	1 1	3.4	5.0
16 hrs	2 hrs	2	1	3.4	[illegible]
20 hrs	2 hrs	2	1	5.4	4.0
24 hrs	2 hrs	2	1	4.0	4.7
2 days	2 hrs	1	1	3.6	4.9
4 days	2 hrs	1	-	4.0	-
7 days	2 hrs	1	1	3.0	3.4

[a] Where more than 1 animal was used, the value given is an average value.

[b] This designation indicates the values obtained from control animals that were injected with NaCl solution at the same time the experimental animals were injected with BAPN solution. The remaining control animals received only H^3TDR injections.

An average of 1256 cells was counted per animal.

was also higher than all the control values obtained over the first 24 hour period with the exception of the 4-hour control animal that had been injected only with H^3TDR.

The range of control values over the first 24 hour period extended from 4.0 percent to 5.4 percent. The values obtained for the percent of cells labeled in the control animals injected with NaCl solution were lower than those of the control animal that were injected only with H^3TDR and sacrificed at the same time periods. However, the difference between the average values for the two types of control animals was only 0.5 percent and was not considered to be a valid difference. The labeling index of the 7-day control animal was the lowest control value obtained (Table 6).

IV. THE EFFECT OF A DISEASE PROCESS UPON THE LABELING INDICIES OF THE CELL POPULATION STUDIED

The values obtained for the percent of cells labeled in the cell populations studied of BAPN-treated rats without evidence of pneumonitis and of BAPN-treated rats with evidence of pneumonitis are given in Table 7.

It can be seen from Table 7 that the percent of cells labeled in the osteogenic layer of periosteum at the site of insertion was greatly depressed in the 20-hour lathyric rats with pneumonitis when compared to the percent of cells labeled in the 20-hour lathyric rat with no evidence of respiratory disease. The percent of cells labeled remained depressed in these animals and the number of labeled cells was so low in the 7-day BAPN-treated rat with respiratory disease that no cell counts were attempted.

TABLE 7- Percent labeled (I_L) cell population in the various tissues studied of BAPN-treated rats of Group II with pneumonitis (A) compared to the values obtained from BAPN-treated rats without pneumonitis (B).

Time sacrificed after injection		Number of animals		Percent of Labeling (I_L)					
BAPN	H^3TDR	Lathyric (A)	(B)	Periosteum (A)	(B)	Ep. Plate (A)	(B)	Adrenal (A)	(B)
20 hrs	2 hrs	2	2	3.9	12.5	12.9[a]	14.6[a]	3.0	5.4
24 hrs	2 hrs	1	2	3.9	14.3	12.6	14.2[a]	3.4	4.0
4 days	2 hrs	1	1	3.1	9.8	12.5	16.0	3.7	4.0
7 days	2 hrs	1	1	-	17.4	6.1	15.3	3.4	3.0

[a] Where 2 animals were used, the value given is an average value.

In contrast to the osteogenic layer of periosteum at the site of muscular insertion, the values obtained for the percent of cells labeled in the proximal tibial epiphyseal plates of BAPN-treated rats with pneumonitis remained about the same as those obtained from BAPN-treated rats without evidence of pneumonitis, and actually fell within the range of values obtained for the 24-hour lathyric rats without respiratory involvement. However, the labeling index was depressed in the epiphyseal plate of the 7-day BAPN-treated rat with pneumonitis.

The percent of cells labeled in the adrenal glands of BAPN-treated rats with pneumonitis did not show the severe depression when compared to the values obtained for comparable lathyric rats with no evidence of pneumonitis, although they were less in all cases except at 7 days.

CHAPTER IV

DISCUSSION

It has been consistently demonstrated that the mucopolysaccharide and collagen fractions of the connective tissue ground substance are somehow affected in lathyrism. These findings have led to a general belief that lathyrogenic agents affect these elements directly and that the connective tissue cell hyperplasia noted at the site of most lathyric lesions is a reactive response secondary to weakening of the connective tissue ground substance. However, it was difficult to interpret the changes in the ground substance without implicating connective tissue cell metabolism, since the connective tissue cells are responsible for the maintainence of the intercellular material. The possibility existed that lathyrogenic agents directly stimulate connective tissue cells to proliferate since an early increase in the number of mitotic figures has been found in the osteogenic layer of periosteum at the site of adductor longus-pectineus insertion in adult lathyric rats (61), and a rather early increase in the number of cells taking up H^3TDR label in the epiphyseal plates of lathyric rats has been reported (65). It was also evident that muscle tension plays an important role in exostosis development since the adductor longus-pectineus exostosis fails to develop when these muscles are sectioned (58). However, it is not definitely

known if the exostosis develops as a result of the combination of muscle tension and weakened connective tissue ground substance or of muscle tension and direct stimulation of the periosteal cells. It was felt that an important positive contribution could be made toward answering the question of whether or not lathyrogens primarily or secondarily cause connective tissue cell proliferation in the periosteum and epiphyseal plates of lathyric rats if the precise time of onset of the connective tissue cell hyperplasia could be determined. Future studies on the precise time that the ground substance is affected in these two areas could then be made and appropriate conclusions concerning the cause of the hyperplasia could be drawn. Utilization of autoradiographic methods using tritiated thymidine label to demonstrate cells preparing for division provided an excellent method of carrying out such an investigation.

Weanling, female albino rats weighing an average of 49 grams were chosen for this investigation because it has been reported that young animals are more susceptible to lathyrogens (7). Therefore, it was felt that the initial reaction of the connective tissue cells would be more severe and easier to measure. In addition, only a limited supply of tritiated thymidine was available and the young rats required a relatively small dose.

During the course of this investigation it was noted that many of the nuclei of the periosteal cells in the osteogenic layer of periosteum of normal animals were quite large and vesicular. No increase

in size of the periosteal cell nuclei was noted until four days after the initial injection of BAPN. These findings are somewhat different than those reported by the author in an earlier paper (87). One of the initial changes in the morphology of periosteal cells under the stimulation of BAPN in that study was a rounding up and enlargement of the normally chromatic and elongated periosteal cell nuclei. The nuclei in the periosteal cells of a 2-day developing exostosis were larger, more vesicular and contained more prominent nucleoli when compared to those in control sections of the osteogenic periosteum at the site of insertion. The difference in these observations is probably due to the fact that the earlier study utilized young adult rats and the periosteal cells in the osteogenic layer of periosteum in these animals were normally not as actively participating in growth as were the periosteal cells in the weanling rats used in the present study. This explanation is partially supported by the finding that the osteogenic periosteum in the 28 day old Group II control animal contained fewer labeled cells than the 21 and 22 day old control animals, and by the work of Tonna and Cronkite (118), who found that the percent of cells labeled in mouse femoral periosteum decreased with age. The latter explanation may also be applied to the extremely early bone formation and early appearance of definite zones in the developing exostoses of this study. The periosteal cells in the young animals were normally more active at the time of BAPN administration, and consequently, did not have to change from a resting to an active state before responding to the BAPN stimulation. Observations by other investigators (9) that indicate an increased severity of lathyric

lesions in rats treated with growth hormone lend additional support to this explanation.

Autoradiographs of the tritiated thymidine-labeled cell populations under investigation showed that the majority of silver grains were directly over the nuclei of the cells that had taken up the label. The rather precise localization of the tritiated thymidine label is primarily due to two reasons. Thymine deoxyribose (thymidine) labeled with tritium (H^3) is a specific DNA precursor and consequently is taken up only by cells that are preparing for mitotic division by synthesizing DNA in order to replicate their chromosomal complement (101). In addition, the beta particle emitted by tritium is a very low energy particle and has an average path length in tissue of about one micron (119). Thus, the beta particles do not penetrate very far from the site of tritium localization. Further attributes of tritiated thymidine as a label for autoradiographic studies and the methods of extracting information from such studies have been previously described in Chapter I.

Autoradiographs of sections of the osteogenic layer of periosteum taken from the approximate center of adductor longus-pectineus insertion of group I control and lathyric rats and of group II control rats and early lathyric rats (up to 12 hours post-BAPN injection) demonstrated the presence of an outer zone of labeled periosteal cells, a central zone containing relatively few labeled cells, and an inner zone containing primarily labeled preosteoblasts. An occasional labeled osteoblast was also noted. The finding of labeled osteoblasts is not unique to this study and has also been reported in

young mouse femoral periosteum (119). Although the cells located in the central zone are apparently not very mitotically active under normal conditions, they do begin to respond to BAPN stimulation approximately 12 hours after the BAPN is given. It would appear that the central zone is an area of "reserve" periosteal cells that can be called upon to divide if the proper stimulus is applied.

Autoradiographs of sections taken from group II 4-and 7-day developing exostoses definitely demonstrated the presence of a proliferative zone as described by Yeager and Gubler (86). In addition, an accumulation of labeled cells was found in the proliferative zone on the medial or pectineus side of sections from 7 and 12-day developing exostoses that were taken from near the center or proximal to the center of insertion. The significance of this finding is not clear since no attempt was made to correlate sites of mitotic activity with growth of the exostosis. However, since Hamre and Yeager (59) have shown that muscle tension is necessary for exostosis formation, the possibility exists that the pectineus muscle is supplying the most tension during exostosis formation and that the periosteal cells are responding to that tension by increased mitotic activity.

Labeled cells were also present in the zone of intercellular material and the zone of intramembranous bone formation in developing exostoses. Thus, attempts at visualizing the migration or displacement of cells from the proliferative zone were relatively unsuccessful. However, conclusions concerning the relative rate of division of the labeled cells present in the three zones could be

inferred from autoradiographs of sections taken from the developing exostoses of group IIIa and IIIb lathyric rats. Following these two groups of animals, it was evident that the cells in the proliferative zone were dividing quite rapidly since the concentrations of silver grains over the nuclei of cells in this zone rapidly diminished with time. Since no parallel rise in the number of labeled cells was noted in the zone of intercellular material, the rapid decrease in the concentration of silver grains over labeled nuclei was probably due to rapid serial dilution of the label by mitotic division and not because the labeled cells all migrated out of the proliferative zone. The label diminished next in the cells located in the zone of intercellular material. And finally, in the 7-day developing exostosis of group IIIa and the 12-day developing exostosis of group IIIb, only occasional labeled osteoblasts and osteocytes were noted.

As mentioned in Chapter II, the experimental rats of group I were injected with BAPN and H^3TDR as near to the same time as possible and then sacrificed at various times up to 20 hours after they were injected. Control animals of this group were injected with H^3TDR and sacrificed at the same time periods. The labeling index of the various cell populations investigated was obtained for each time period by taking the ratio of the number of labeled cells counted (N_2) to the total number of cells counted (N) and multiplying the resulting decimal by 100. Therefore, the labeling index and the per cent of cells labeled are synonymous terms as used in this paper. Injecting the experimental animals with BAPN and H^3TDR at the same

time and sacrificing the animals serially up to 20 hours after the injections permitted the examination of the effect of BAPN upon the rate at which the various cell populations passed from the period of DNA synthesis (T_s) through the post-synthetic rest period (G_2) and the period of mitotic division (M) (Text Figure 1, Chapter I). It was felt that if BAPN affected the rate at which the cell populations studied passed through these three periods of the cell generative cycle, then the per cent of cells labeled in the BAPN-treated cell populations would show some deviation from the per cent of cells labeled in control populations during the time periods studied. It was found that the per cent of cells labeled in the osteogenic periosteum at the site of insertion and in the proximal tibial epiphyseal plates increased with time more rapidly than the per cent of cells labeled in control animals. The per cent of cells labeled in the osteogenic layer of periosteum began to increase above control values 8 hours after injections were given. However, the range of values obtained for experimental and control animals did not separate completely until 12 hours after the injections were given. The per cent of cells labeled in the epiphyseal plates of group I experimental animals also began to increase above control values 8 hours after the injections were given and remained above control values at 12 hours and 16 hours after the injections were given. The only overlap of experimental and control values during these three time periods was noted at 16 hours. These findings indicated that the BAPN-stimulated cells in the osteogenic layer of periosteum and proximal epiphyseal plate were passing more

rapidly through the period of the cell generative cycle extending from the period of DNA synthesis through mitotic division.

Tonna and Cronkite (106) have indicated that if the time after administration of the H^3TDR to sacrifice is not shorter than the minimum time of post-synthetic rest of about one hour, an enlargement of the labeled population could be expected since some labeled cells would have divided. Thus, a gradual increase in the percent of cells labeled should occur with increasing time after H^3TDR injection. If an average of the values obtained for the group II control animals that were sacrificed during the initial 24 hours studied is used as the 2 hour post-H^3TDR value, then the percent of cells labeled for the osteogenic layer of periosteum in control animals at 1 hour, 2 hours, 4 hours and 8 hours post-H^3TDR injection was 4.9 percent, 7.6 percent, 8.4 percent and 9.1 percent respectively. The control values for the percent of cells labeled in the proximal epiphyseal plates at the same times was 7.6 percent 9.4 percent, 14.2 percent and 14.5 percent respectively. In addition, if the work of Young (120) on the generative cycle of cells in young rat periosteum is examined, it can be seen that the labeled population should increase by about 80 percent at 12 hours after the injection of H^3TDR. The osteogenic periosteum at the site of insertion in control animals of group I showed an increase of 90 per cent at 12 hours after H^3TDR injection. The values obtained for the percent of cells labeled in the epiphyseal plates of group I control animals showed that a 100 percent change occurred between 8 and 12 hours after H^3TDR injection. This finding

correlates well with what could be expected from other rapidly proliferating cell systems (103).

The cell populations investigated in the adrenal cortex of group I experimental animals showed no consistent change during the time period studied.

The percent of cells labeled in the cell populations of the osteogenic periosteum, proximal tibial epiphyseal plate and zona glomerulosa (plus transition zone) of the group I 20-hour lathyric rat was less than the control values obtained for these three cell populations. This finding was a deviation from the trend of increased experimental values observed in the osteogenic periosteum and epiphyseal plates. The reason for this deviation from the trend are not clear. However, the consistency of this finding in three cell populations would seem to indicate a general depression of mitotic activity in the one lathyric rat examined at this time.

The experimental animals of group II were initially injected with BAPN and then injected with H^3TDR 2 hours prior to sacrifice. The experimental animals of group II essentially tested the effect of BAPN on the percent of cells of the total population studied that were in the DNA synthesis period of the cell generative cycle at progressively longer periods of time after BAPN injection. The control animals were injected with NaCl solution to insure that the observed changes were not due to the injection alone. The initial change in the osteogenic layer of periosteum and proximal tibial epiphyseal plates of group II BAPN-treated rats was a decrease in the percent of labeled cells. The decrease was evident in the

osteogenic layer of periosteum at 4 and 8 hours after the injection of BAPN in the proximal tibial epiphyseal plate. Since the labeled cells present at 4 hours and 8 hours were labeled within a few minutes after the H^3TDR injection given 2 hours prior to sacrifice, the decrease in percent of cells synthesizing DNA had occurred by 2 hours post-BAPN injection in the osteogenic layer of periosteum and by 6 hours post-BAPN injection in the proximal tibial epiphyseal plate. The explanation for this decrease remains to be elucidated. It was apparent that the mechanical stress caused by the injection of BAPN did not cause the decreased values since control animals that had been injected with NaCl solution did not show a decrease in the percent of cells labeled in these two areas. At least two possible explanations remain. BAPN could presumably directly depress the metabolic activity of connective tissue cells by inhibiting some enzyme system that normally plays an active role in maintaining the functions of the connective tissue cells. If the normal route of metabolism is blocked, an alternative metabolic pathway could possible be activated that might be responsible for the altered activity of the connective tissue cells at these two sites. The other possibility is that BAPN either initially causes an increased release of cortisone or sensitizes the affected tissues to the effect of cortisone. It has been shown by various authors that cortisone inhibits the production of lathyric lesions (9, 221). It has been found that cortisone depresses connective tissue cell proliferation and also inhibits the formation of connective tissue fibers and ground sub-

stance (122). Either of the proposed explanations for the initial depression of the percent of cells labeled in the osteogenic periosteum and the proximal tibial epiphyseal plates could cause a weakening of extracellular connective tissue elements and thereby cause the increased mitotic activity observed by 12 hours post-BAPN injection in the osteogenic periosteum at the site of insertion and by 20 hours post-BAPN injection in the proximal tibial epiphyseal plates. Again, the increase in the percent of cells labeled was probably present 2 hours earlier than indicated by the data since the H^3TDR injection was given 2 hours prior to sacrifice. Therefore, before any definite conclusions can be drawn concerning the reaction of the connective tissue cells to weakening of the ground substance in the osteogenic periosteum and proximal tibial epiphyseal plates of lathyric rats definite proof must be obtained that indicates a change in the extracellular elements of the connective tissue before 14 hours after BAPN treatment in the osteogenic layer of periosteum and before 14 hours after BAPN treatment in the proximal epiphyseal plates.

The difference in the time of onset of the noted increase in labeled cells between the osteogenic periosteum and proximal tibial epiphyseal plate is probably best explained by the vascularity of the two areas involved. The epiphyseal plate is avascular. Consequently, the factor responsible for the cellular proliferation would have to diffuse into the cartilage and thus a longer period of time would elapse before the cartilage cells were affected.

It has been mentioned in Chapter I that the labeling index ap-

proximately measures the percentage of total generation time occupied by the period of DNA synthesis. The increased indicies of labeling found in the osteogenic layer of periosteum and proximal tibial epiphyseal plate indicate that either the DNA synthesis time has increased and the total generative time has remained constant, or that the generative time has decreased and the DNA synthesis time has remained constant. The possibility also exists that both the synthesis time and total generative time have changed in some manner. It has been pointed out that the pre-synthetic rest period is the most variable period in cell systems that divide upon demand (i.e. periosteum). Thus, the pre-synthetic rest period could be expected to be shorter in BAPN-treated rats. In addition, evidence from group I indicated that the labeled cell population in the osteogenic layer of periosteum and proximal tibial epiphyseal plate pass more rapidly from the period of DNA synthesis through mitotic division in BAPN-treated rats. Therefore, it would appear that the entire generative cycle is speeded up in these two sites in lathyric rats. However, it is suggested that an important contribution would be made to the illucidation of the affect of BAPN upon these two cell populations if the precise changes in the generative cycle were known.

BAPN appeared to decrease the percent of cells labeled in the zona glomerulosa and transition zone of the rat adrenal cortex. The values obtained for BAPN-treated rats were less than control values with the exception of the 20-hour lathyric rat. Because of this value, it is felt that the results obtained for the adrenal

should be interpreted with caution. A source of error inherent in studies of this kind lies in the adequacy of the sampling methods used. This is especially true when the differences observed are in the vicinity of one percent. However, it can be said that no measureable hyperplasia of the zona glomerulosa or transition zone occurred during the course of this experiment as might have been expected from some reports in the literature (89, 90, 91). It is interesting to note that the values obtained for the percent of cells labeled in the zona glomerulosa and transition zone of lathyric rats with respiratory disease were all somewhat lower than the values obtained for the same cell populations in lathyric rats without respiratory disease. It seems quite certain that a pneumonitis would be a source of systemic stress. Therefore, the possibility certainly exists that the adrenal cortex might respond to a stressful condition by decreasing mitotic activity, at least temporarily. However, if this is the case, the finding of severe depression in the percent of cells labeled in the osteogenic layer of periosteum and the less severe depression of the percent of cells labeled in the proximal tibial epiphyseal plate of lathyric rats with pneumonitis is in conflict with the work of Selye (9) who found that stress enhanced the action of lathyrogens. It is evident that more work should be done on the effect of stress upon the initial changes in the proliferative pattern of adrenal cortical cells.

This study has raised many new questions that should be answered. Some of the possibilities for new research in lathyrism

have already been suggested, but will be repeated here. The entire generative cycle of BAPN-stimulated cells in the osteogenic layer of periosteum and proximal epiphyseal plate should be investigated in an attempt at determining the precise alteration that occurs. An interesting study on the regression of lathyric lesions utilizing autoradioraphy and H^3TDR could be done. More information is needed on the correlation of sites of mitotic activity and bone formation in developing exostoses as well as on the migration or displacement of cells from the proliferative zone into the other two zones found in a developing exostosis. The finding that a disease process severely affects the rate of mitotic division in the osteogenic periosteum may have some important implications in relation to human disease and bone formation and should be pursued further. Further studies on the initial metabolic changes in cells affected by lathyrogens should be carried out. The effect of stressful conditions on cell proliferation in the adrenal cortex should be made and compared to the effect of BAPN. And finally, an attempt should be made at determining the precise time that connective tissue ground substance is affected in lathyrism.

Chapter V

SUMMARY AND CONCLUSIONS

A total of 58 weanling albino rats was used in an autoradiographic study of the effect of BAPN upon the distribution and percent of tritiated thymidine-labeled cells in the osteogenic layer of periosteum at the site of adductor lengus-pectineus insertion, the proximal tibial epiphyseal plate and adrenal cortex. Lathyrism was induced in the experimental animals by giving daily intraperitoneal injections containing 30 milligrams of BAPN in 1 milliliter of distilled water. Cell populations synthesizing DNA in experimental and control animals were labeled by giving intraperitoneal injections of tritiated thymidine (H^3 TDR, sp. Act. 1.9 c/mM, Schwarz Bioresearch Inc.) at a dosage of 0.5 microcuries per gram of body weight. Animals were sacrificed by giving an overdose of sodium pentobarbitol. All tissues were fixed in Bouin-Hollande fixative for three days. Bone was decalcified in a 5 percent solution of ethylenediaminetetraacetic acid. Paraffin sections were cut at 6 micra and mounted on chemically clean glass slides. A routine hematoxylin and eosin stain was performed on samples of all tissues. Slides to be used for autoradiography were either stained with the periodic-Schiff reaction before dip-coating with Nodak NTB2 nuclear emulsion and stained with hematoxylin alone. The emulsion was exposed from 30 to 50 days in a cold, dry atmosphere, and then developed (all solutions at 17°C.)

in undiluted Kodak Dektol for 2 minutes, rinsed with water, fixed in "hypo" for 15 minutes, washed in running tap water for 1 hour, dehydrated, passed through 2 changes of xylene, mounted in permount and coverslipped. The percent of cells labeled (I_L) in the cell populations studied was determined by the equation $I_L = N_2/N \times 100$, where N_2 is equal to the number of labeled cells and N is equal to the total number of cells counted. Three types of experiments were run.

The experimental animals of Group I were simultaneously injected with BAPN and H^3TDR and sacrificed at 1 hour, 4 hours, 8 hours, 12 hours, 16 hours and 20 hours after the injections. Control animals were injected only with H^3TDR and sacrificed at the same time periods. This experiment was a test of the effect of BAPN on the rate at which the investigated cell populations passed through that portion of the cell generative cycle extending from the period of DNA synthesis through the period of post-synthetic rest and actual mitotic division.

The experimental animals of Group II were injected with BAPN and then injected with H^3TDR 2 hours before they were sacrificed at 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, 24 hours, 2 days, 4 days, and 7 days after the initial injection of BAPN. Three control animals were injected with sodium chloride solution and then injected with H^3TDR before being sacrificed with the 4 hour, 8 hour, and 12 hour BAPN-treated rats. The remaining control rats received only H^3TDR injections and were sacrificed with the 4 hour, 8 hour, 12 hour, 16 hour, 20 hour, 24 hour, 2 day, and 7 day experimental animals.

This experiment tested the effect of BAPN on the percent of labeled cells present in the cell populations studied at the stated time periods after BAPN injection.

The animals of Group III were divided into Group IIIa and IIIb for descriptive purposes. The experimental animals of Group IIIa were injected with H^3TDR 24 hours after the initial injection of BAPN and sacrificed at 2 days, 4 days and 7 days after the initial injection of BAPN. Control animals were injected only with H^3TDR and sacrificed with the 2 day and 7 day experimental animals. The experimental animals of Group IIIb were injected with H^3TDR 4 days after the initial injection of BAPN and sacrificed at 7 days, 9 days and 12 days after the initial injection of BAPN. The 12-day lathyric rat received an additional injection of H^3TDR 2 hours prior to sacrifice. Group III was designed primarily to trace the origin and migration or displacement of the various cell types present in developing exostoses.

The labeled cells in the osteogenic layer of periosteum near the center of the adductor longus-pectineus insertion of Group I experimental and control animals were found to be distributed in an outer zone of labeled periosteal cells located immediately subjacent to the fibrous layer of periosteum and an inner zone of labeled cells that were predominately preosteoblasts located immediately above the cortical bone of the femur. A central zone containing relatively few labeled cells was located between the outer and inner zones. The inner and outer zones could be seen to merge at the medial and lateral peripheries of the insertion and also proximal and distal to the center of insertion.

The same distribution was noted in all Group II control animals and in the Group II experimental animals up to 12 hours after BAPN injection. At that time the central zone became progressively more heavily labeled until the labeled cells in the osteogenic layer of periosteum appeared to be randomly distributed. The majority of labeled cells in the Group II 4-day developing exostosis were located in a proliferative zone located between the fibrous layer of periosteum and the zone of intercellular material. The same was true of the 7-day and 12-day developing exostoses but a large portion of the labeled cells appeared to be concentrated on the medial side of the developing exostoses in sections taken from the center or proximal to the center of insertion. Labeled cells were also found to be present in the zone of intercellular material and zone of intramembranous bone formation. Autoradiographs of sections taken from the developing exostoses of Group IIIa and IIIb experimental animals showed that the label was most rapidly diluted by mitotic division in the proliferative zone, than in the zone of intercellular material, while in the zone of intramembranous bone formation an occasional comparatively well labeled osteoblast or osteocyte could still be found.

The labeled cells in the proximal tibial epiphyseal plate were localized to the zone of proliferating cartilage cells in control animals that had been sacrificed up to 16 hours after the injection of H^3TDR. At 16 hours post-BAPN injection some labeled cells were located in the zone of maturing cartilage cells and this downward displacement of cells became more evident with time. No difference in the distribution of

labeled cells in the lathyric epiphyseal plates was noted although a slight disorganization of the epiphyseal plates was first noted in the 7-day lathyric rats.

The majority of labeled cells present in the adrenal cortex were located in the zona glomerulosa and transition zone in Group I and Group II control and experimental animals.

It was found that treatment with BAPN increased the rate at which labeled cells passed through the portion of the cell generative cycle extending from the period of DNA synthesis through the post-synthetic rest period and the period of mitotic division in the osteogenic layer of periosteum at the site of insertion and in the proximal tibial epiphyseal plates. The increase in rate was manifested as an increase in the percent of cells labeled in BAPN-treated animals over control values by 12 hours in the osteogenic periosteum and by 8 hours in the proximal epiphyseal plate. No consistent trend was noted in the zona glomerulosa (including the transition zone) or outer portion of the zona fasciculata of the adrenal cortex.

It was also found that treatment with BAPN caused an initial decrease in the percent of cells labeled in the osteogenic layer of periosteum and proximal tibial epiphyseal plate in Group II experimental animals. The decrease was evident in the osteogenic layer of periosteum of the 4-hour and 8-hour lathyric rats and in the epiphyseal plate of the 8-hour lathyric rat. The values obtained for the percent of cells labeled in lathyric rats then increased to become higher than control values by 12 hours in the osteogenic layer of periosteum and by 20 hours in the proximal tibial epiphyseal plate. Treatment with BAPN

appeared to decrease the percent of cells labeled in the zona glomerulosa (including the transition zone), but it was urged that these results be interpreted with caution because of the low magnitude of the observed changes and because of one apparently aberrant high value obtained 20 hours after the injection of BAPN.

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PLATE I

Figure 1. Photomicrograph of a section of lung taken from a Group II 7-day lathyric rat with evident pneumonitis. (Hematoxylin and Eosin)X420

Figure 2. Photomicrograph of a section of lung taken from a Group II 4-day control rat with pneumonitis. (Hematoxylin and Eosin) X420

Figure 1

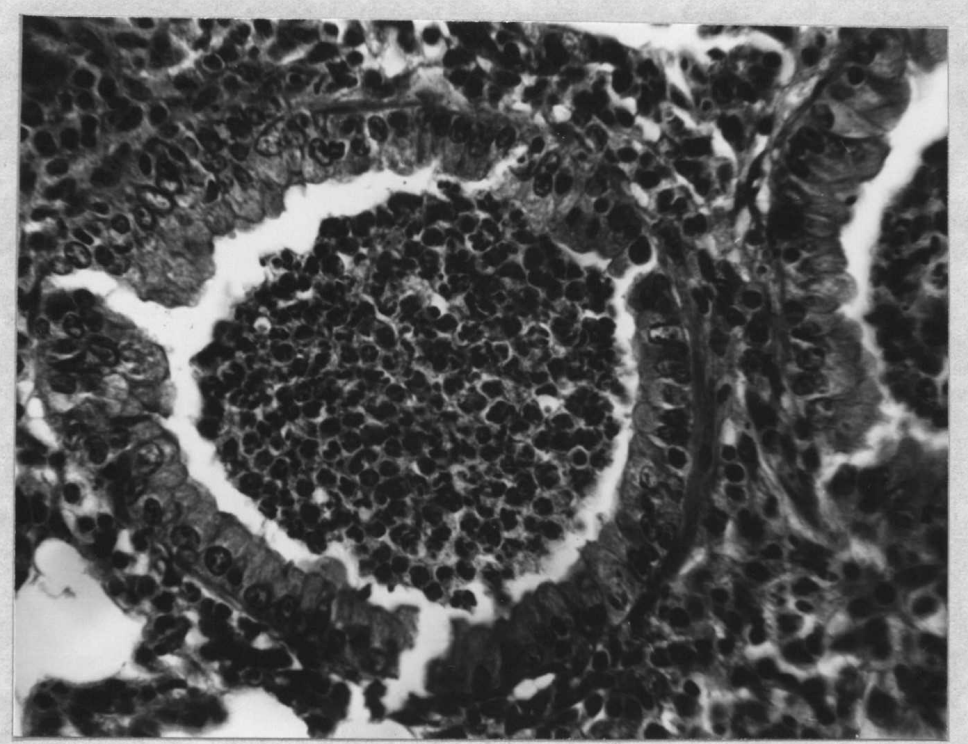

Figure 2

PLATE I

PLATE II

Figure 3. Photomicrograph of a section of lung taken from a 12-hour lathyric rat without respiratory disease. (Hematoxylin and Eosin) X420

Figure 4. Photomicrograph of the periosteum from the common site of adductor longus and pectineus insertion of a normal rat. F.L., Fibrous layer of periosteum; O.L., Osteogenic layer of periosteum; 1., vesicular periosteal cell nuclei; 2., Preosteoblast; 3., Osteoblast. (Hematoxylin and Eosin) X101.

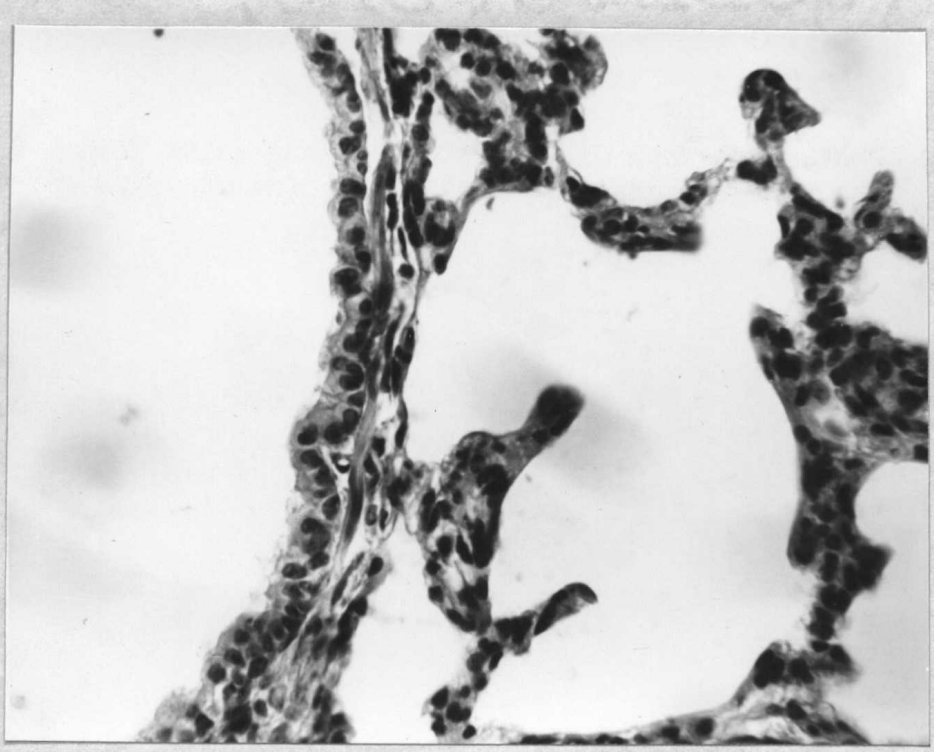

Figure 3

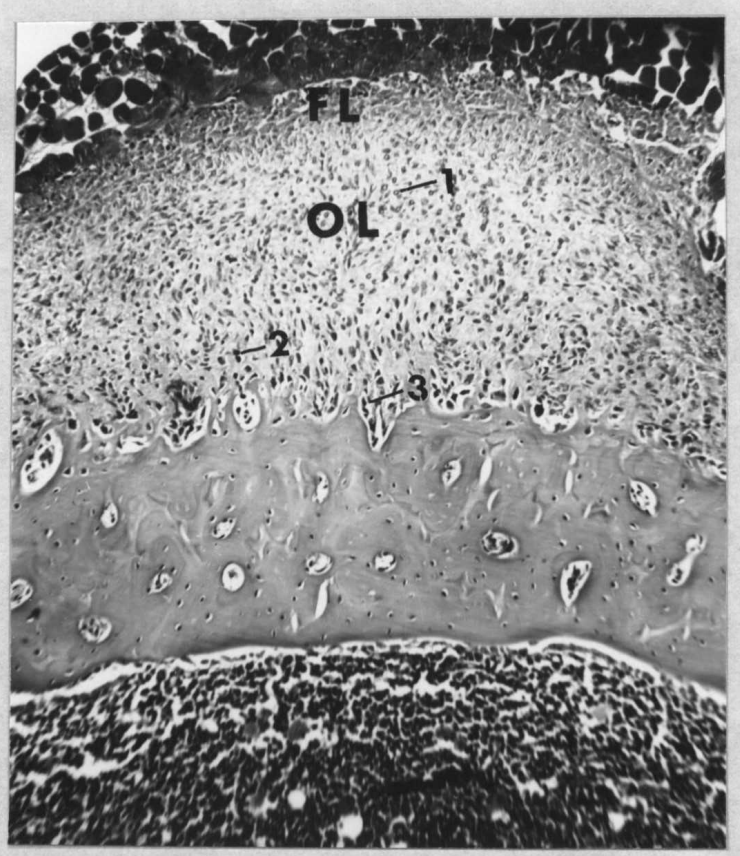

Figure 4

PLATE II

PLATE III

Figure 5. Photomicrograph of the osteogenic layer of periosteum from the comm
site of adductor longus-pectineus insertion of a normal rat.
1., periosteal cell ; 2., preosteoblast; 3., osteoblast; F., Femur
(Hematoxylin and Eosin) X420.

Figure 6. Photomicrograph of a section taken from a Group II 4-day developing
exostosis. I.M, intercellular material; N.B., new bone.
(Hematoxylin and Eosin) X101.

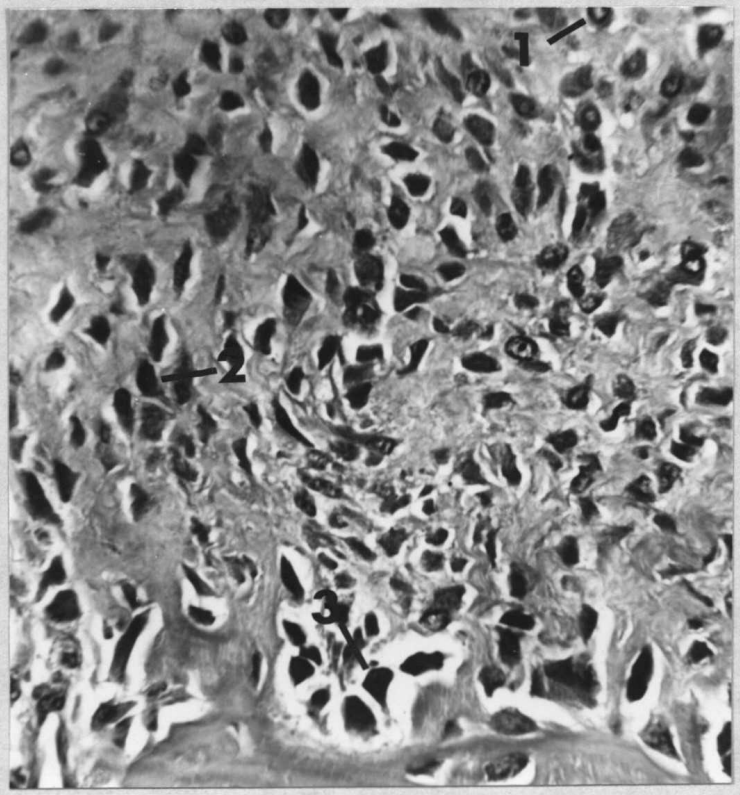

Figure 5

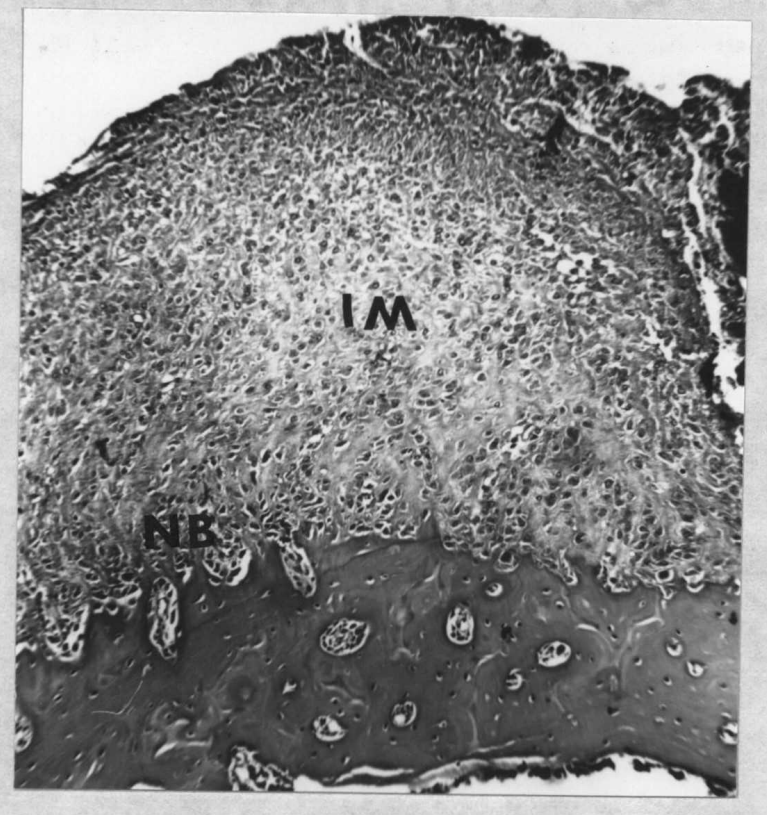

Figure 6

PLATE III

Figure 7. Photomicrograph of an autoradiograph of a Group II 4-day developing exostosis. P.Z., proliferating zone sampled for percent of cells labeled. Arrows indicate labeled cells. (Periodic Acid-Schiff and Hematoxylin) X101.

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Figure 8. Photomicrograph of an autoradiograph of a Group II 7-day developing exostosis. P.Z., proliferative zone sampled for the percent of cells labeled. The dark spots are labeled cells. (Periodic Acid-Schiff) X50.

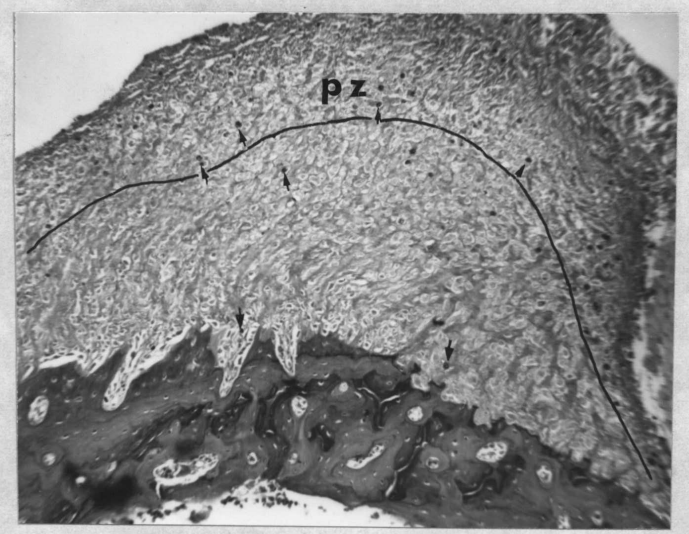

Figure 7

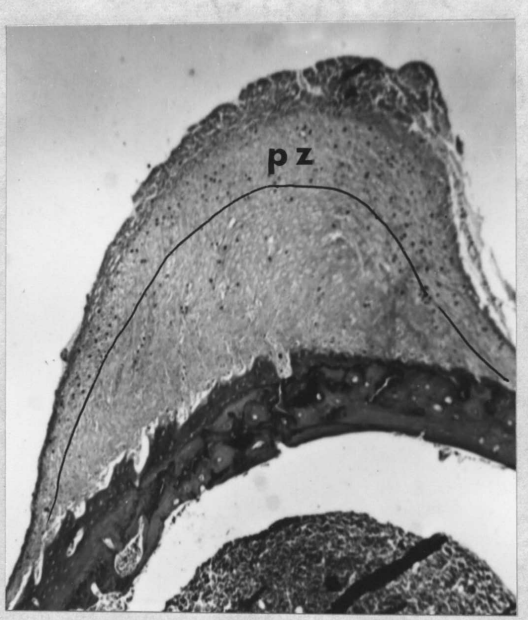

Figure 8

PLATE IV

PLATE V

Figure 9. Photomicrograph of a section from the proximal epiphyseal plate of a normal rat. The lines indicate the upper and lower boundries of the area sampled for labeled cells. 1., zone of resting cartilage; 2., zone of proliferating cartilage; 3., zone of maturing cartilage; 4., zone of calcifying cartilage. (Hematoxylin and Eosin) X180.

Figure 10. Photomicrograph of adrenal cortex from a normal animal. Areas 2 and 3 were sampled as one population. The bottom line represents the lower boundry of the fascicular area sampled for labeled cells. 1., capsule; 2., zona glomerulosa ; 3., transition zone; 4., zona fasciculata. The zona reticulosis is not shown. (Hematoxylin and Eosin) X420.

Figure 9

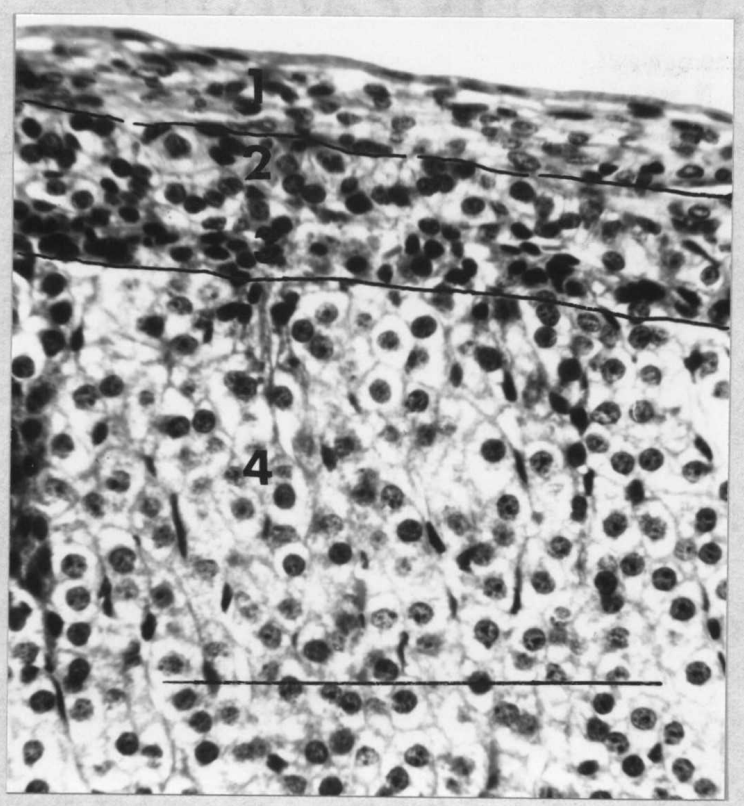

Figure 10

PLATE V

PLATE VI

Figure 11. Photomicrograph of the osteogenic layer of periosteum from a Group II 2-day lathyric exostosis. Hematoxylin and Eosin. P., periosteal cells; I.M., intercellular material; N.B., new bone. (Hematoxylin and Eosin) X420.

Figure 12. Photomicrograph of periosteal cells from a Group II 4-day developing exostosis. Compare with Figure 11 for increase in size of nuclei and nucleoli. 1., periosteal cells; 2., mitotic figure; 3., intercellular material. (Hematoxylin and Eosin) X420.

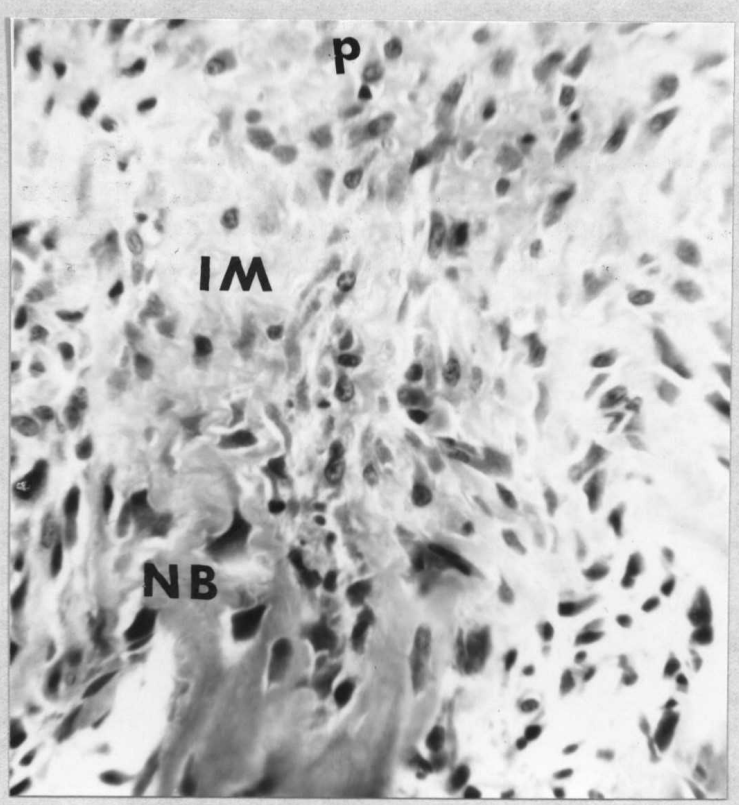

Figure 11

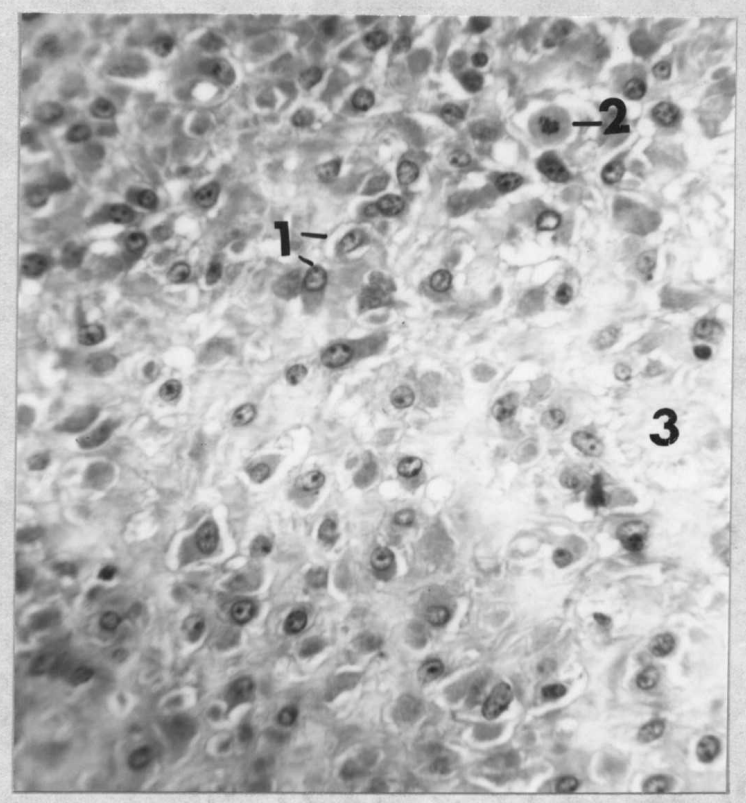

Figure 12

PLATE VI

Figure 13. Photomicrograph of a 4-day developing exostosis. P.Z., proliferative zone; I.M., zone of intercellular material; N.B., zone of intramembranous bone formation. (Hematoxylin and Eosin) X101.

Figure 14. Photomicrograph of a 9-day developing exostosis. P.Z., proliferative zone; I.M., zone of intercellular material; N.B., zone of intramembranous bone formation (Hematoxylin and Eosin) X101.

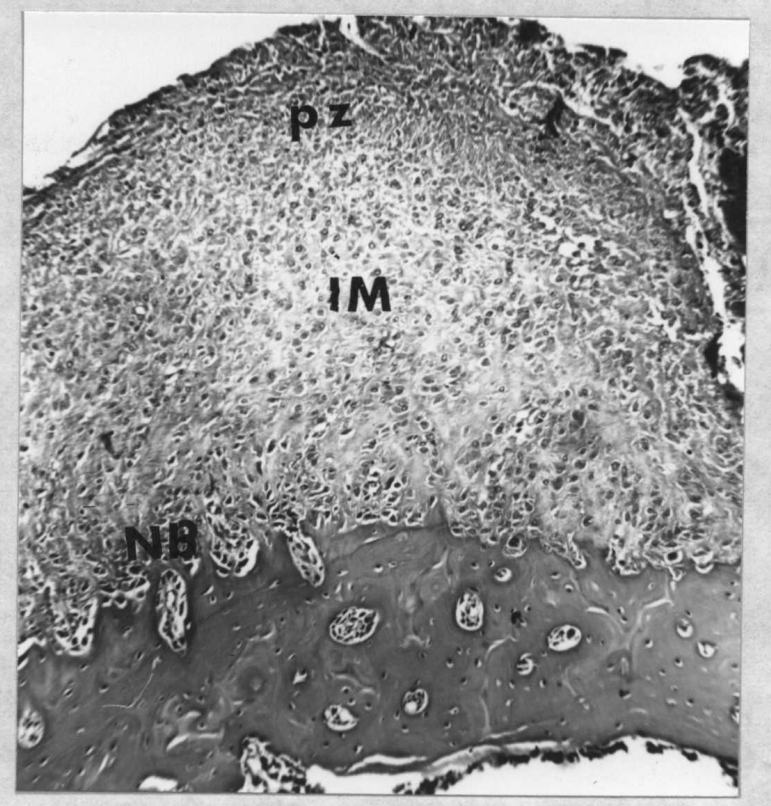

Figure 13

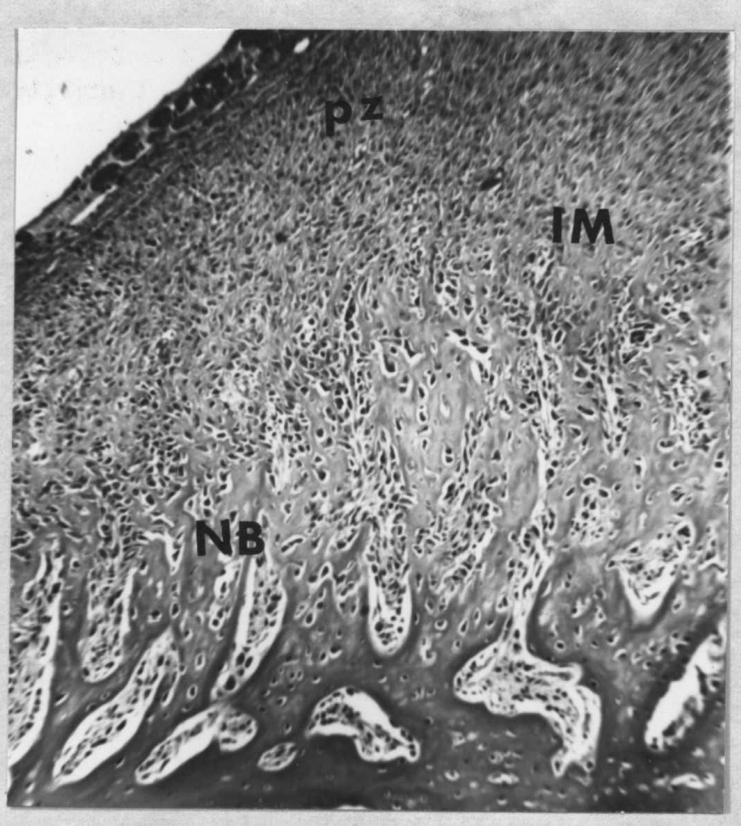

Figure 14

PLATE VII

Figure 15. Photomicrograph of 7-day developing exostosis from a rat with pneumonitis. Note the large amount of intercellular material and the lack of a proliferative zone and zone of intramembranous bone formation. Compare with Figure 8. (Hematoxylin and Eosin) 101X.

Figure 16. Photomicrograph of an autoradiograph of normal periosteum from a Group II control animal. The dark line separates the fibrous layer (F.L.) from the osteogenic layer (O.L.) of periosteum. Note the distribution of labeled cells (arrows) in the osteogenic layer of periosteum in an outer zone of labeled cells and an inner zone of labeled cells. The O.L. is printed in the sparsely labeled central zone. X101.

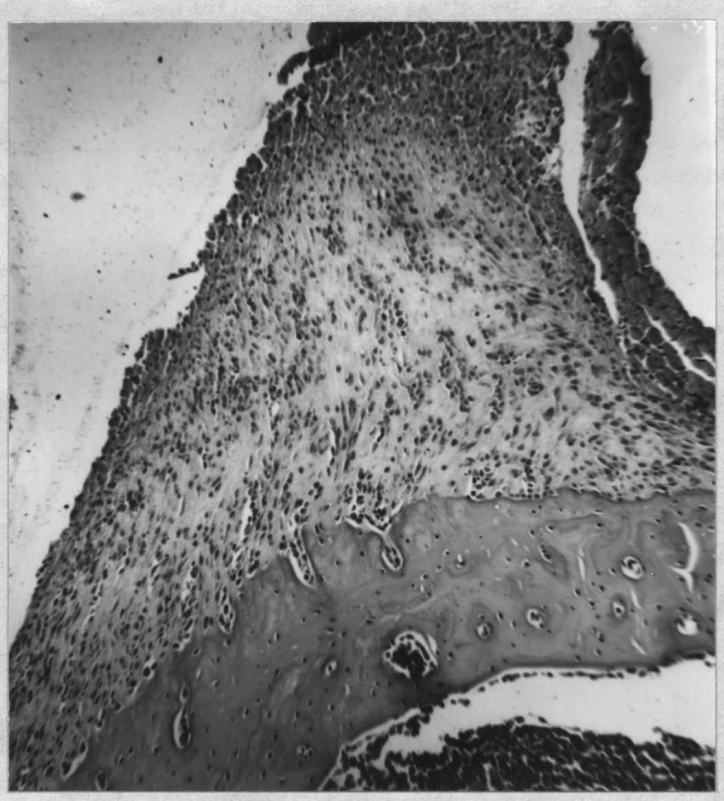

Figure 15

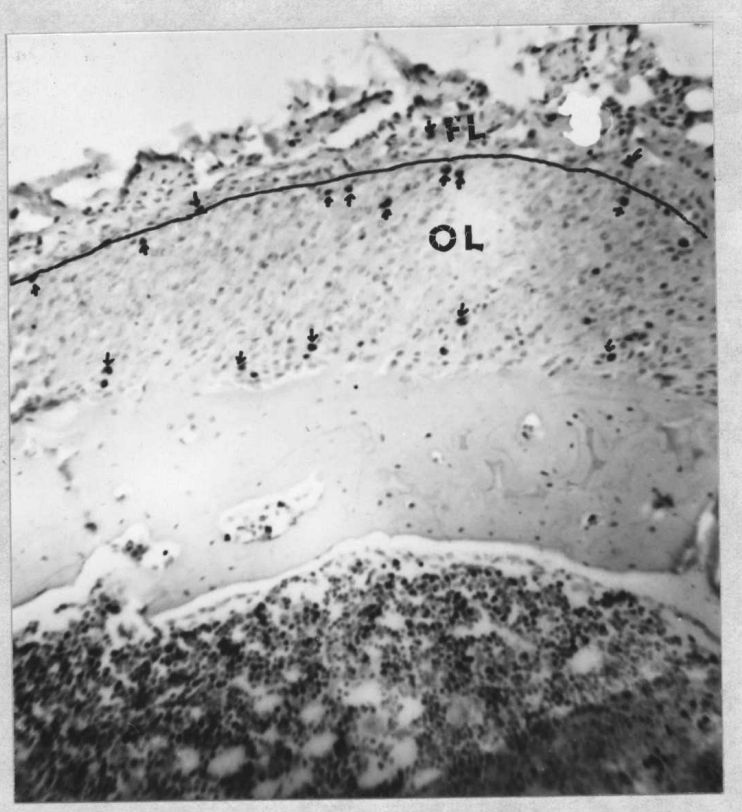

Figure 16

PLATE VIII

PLATE IX

Figure 17. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum from a Group II control animal. 1., labeled osteoblast; 2., labeled preosteoblast. The generally sparsely labeled central zone contains some labeled cells in this photomicrograph, which was not unusual near the peripheries of the insertion. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 18. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum from a Group I control animal sacrificed 20 hours after H^3TDR injection. 1., outer zone of labeled cells; 2., central zone containing relatively few labeled cells; 3., inner zone of labeled cells. (Periodic Acid-Schiff and Hematoxylin X420.

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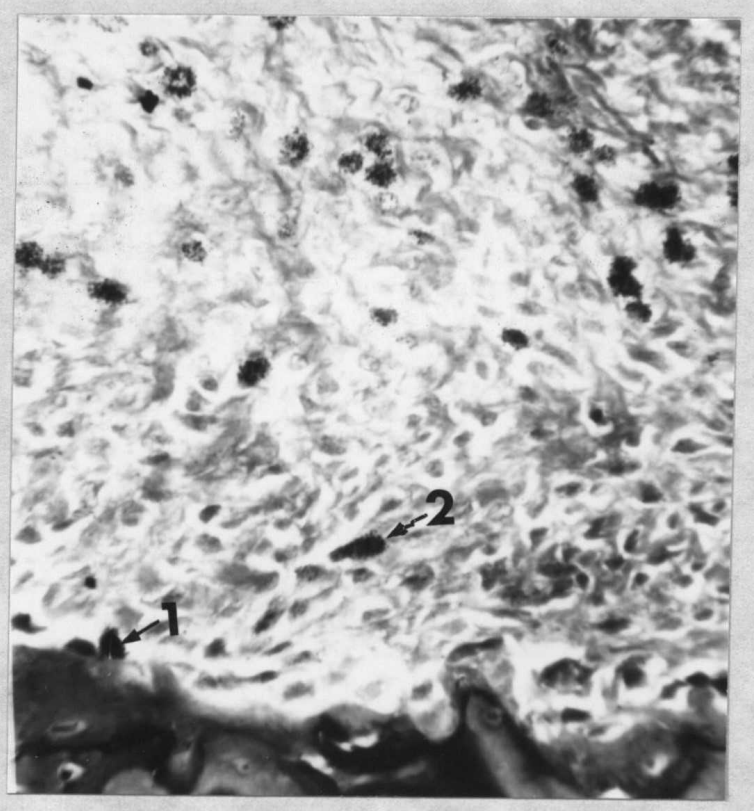

Figure 17

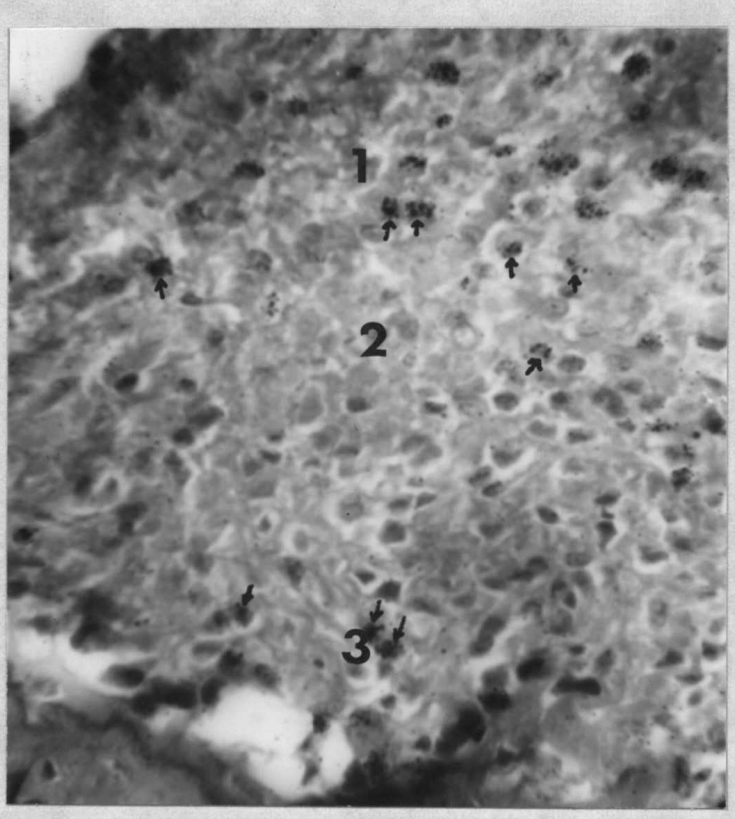

Figure 18

PLATE IX

PLATE X

Figure 19. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum from a Group III control animal killed 16 hours after H^3TDR injection. The photomicrograph was taken from near the periphery of insertion. Note the prescence of labeled cells in all areas of the osteogenic periosteum also see Figure 17. F.L., fibrous layer; O.L., osteogenic layer. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 20. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum distal to the center of adductor longus-pectineus insertion from a Group III control animal killed 20 hours after H^3TDR injection. Note the random distribution of cells in the osteogenic layer of periosteum. The cloudy areas in the photomicrograph are due to the uneven thickness of the emulsion covering the section. (Periodic Acid-Schiff and Hematoxylin) X420.

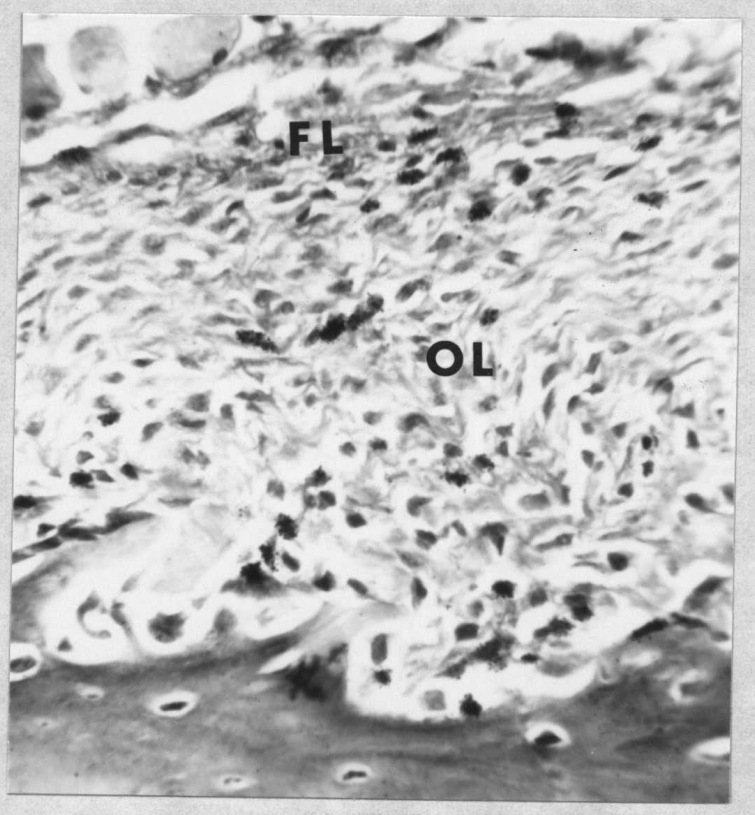

Figure 19

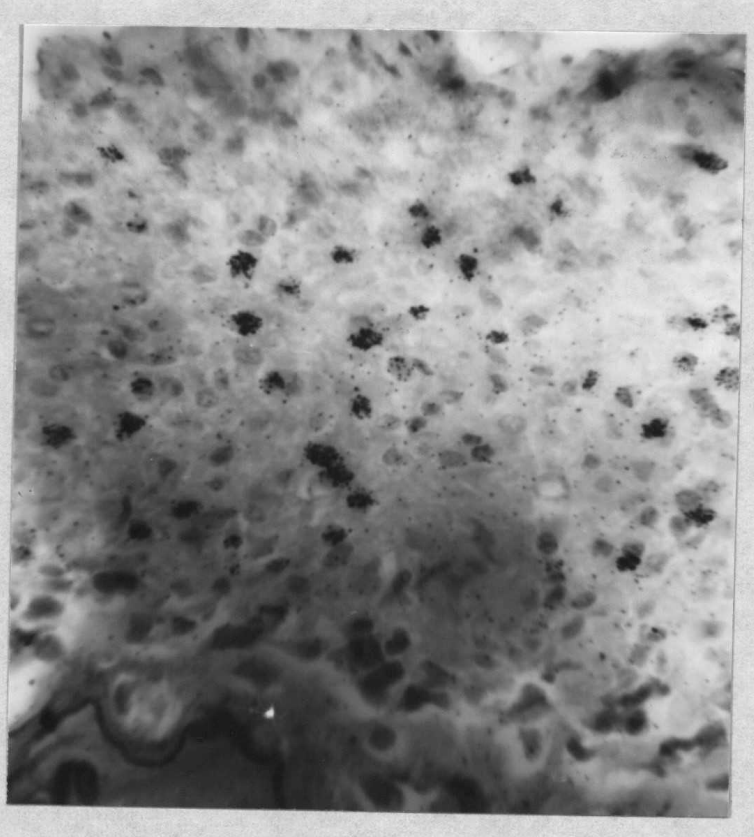

Figure 20

PLATE X

PLATE XI

Figure 21. Photomicrograph of a diagrammatic drawing illustrating the five sections sampled for the percent of labeled cells and the distribution of labeled cells in a Group III control animal sacrificed 16 hours after H^3TDR injection. Section A & B were taken distal to the center of adductor longus-pectineus insertion, section C-from the center of insertion and sections D & E were taken proximal to the center of insertion. The stippling represents the relative distribution of labeled cells in the osteogenic periosteum. Only the short medial portion of the pectineus muscle (P) is diagramed. A., adductor longus.

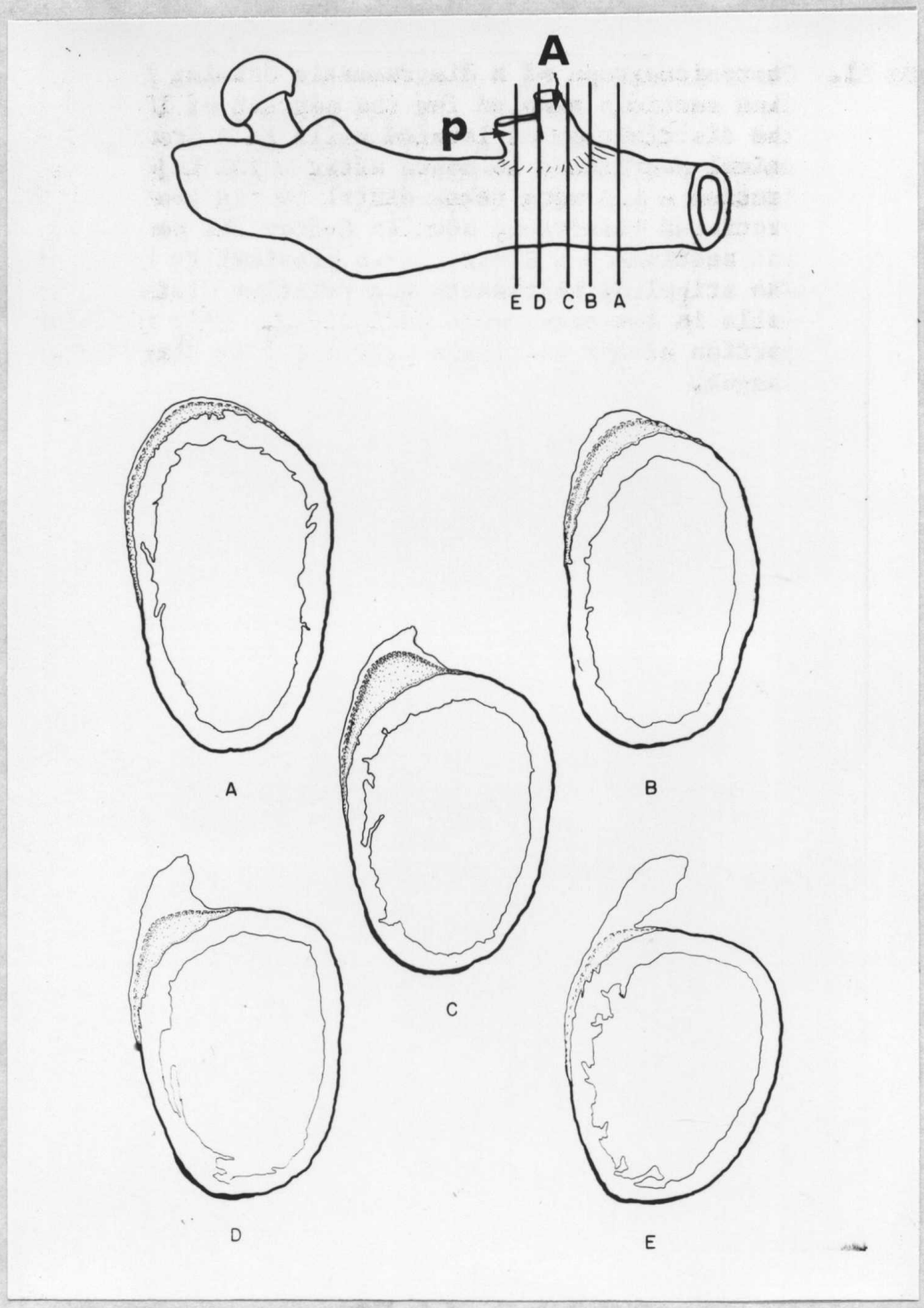

Figure 21

PLATE XI

PLATE XII

Figure 22. Photomicrograph of an autoradiograph of the osteogenic periosteum from a group IIIa control animal sacrificed 6 days after the injection of H^3TDR. The arrows indicate a labeled osteoblast and a labeled osteocyte. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 23. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum from a 16-hour lathyric rat of group I. Note that the 3 zones are evident. 1., outer zone of labeled cells; 2., central zone containing relatively few labeled cells; 3., inner zone of labeled cells. (Periodic Acid-Schiff and Hematoxylin) X420

Figure 22

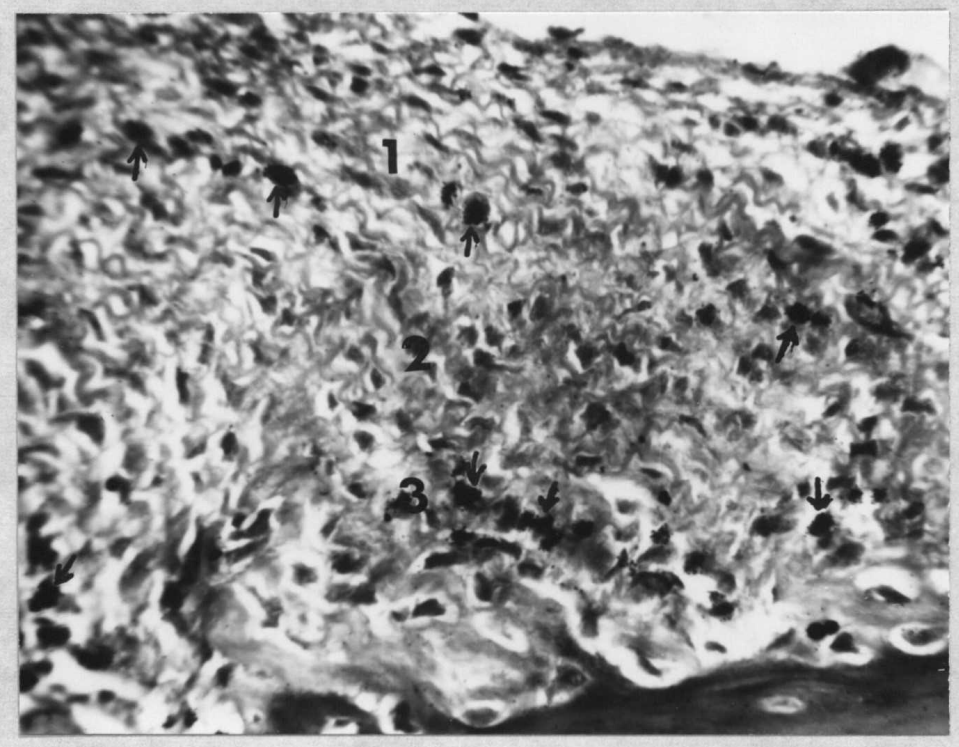

Figure 23

PLATE XII

PLATE XIII

Figure 24. Photomicrograph of an autoradiograph of the osteogenic periosteum from a 12-hour lathyric rat of group II. The arrows indicate that the labeled cells are randomly distributed throughout the osteogenic layer of periosteum. (Periodic Acid-Schiff and Hematoxylin) X101.

Figure 25. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum from a group II 2-day lathyric rat showing labeled cells in the central zone. (Periodic Acid-Schiff and Hematoxylin) X420.

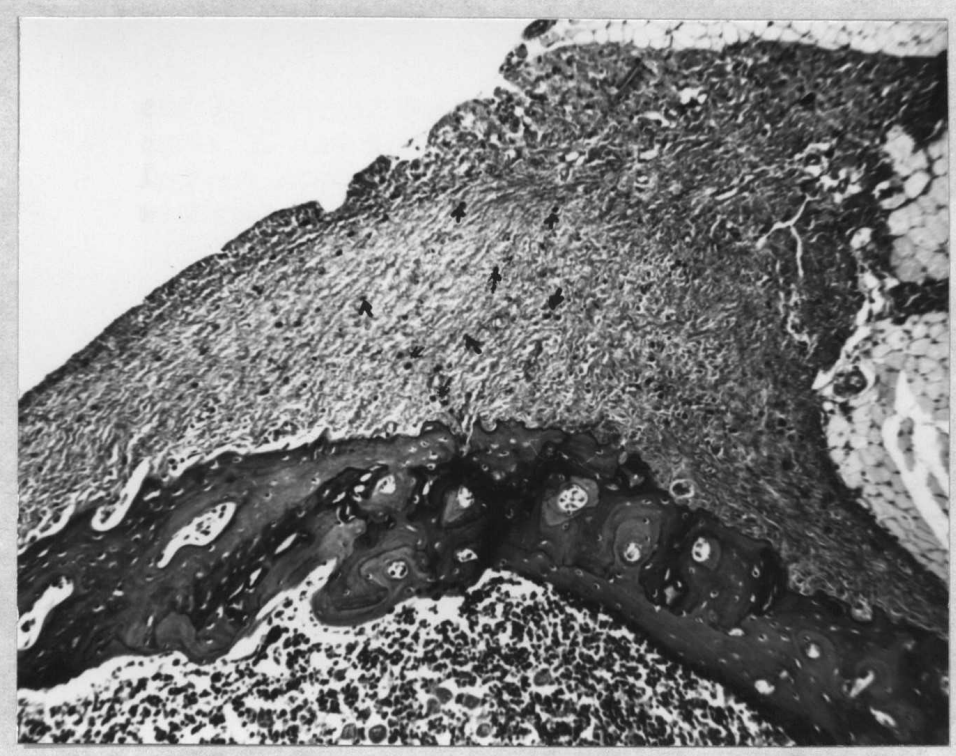

Figure 24

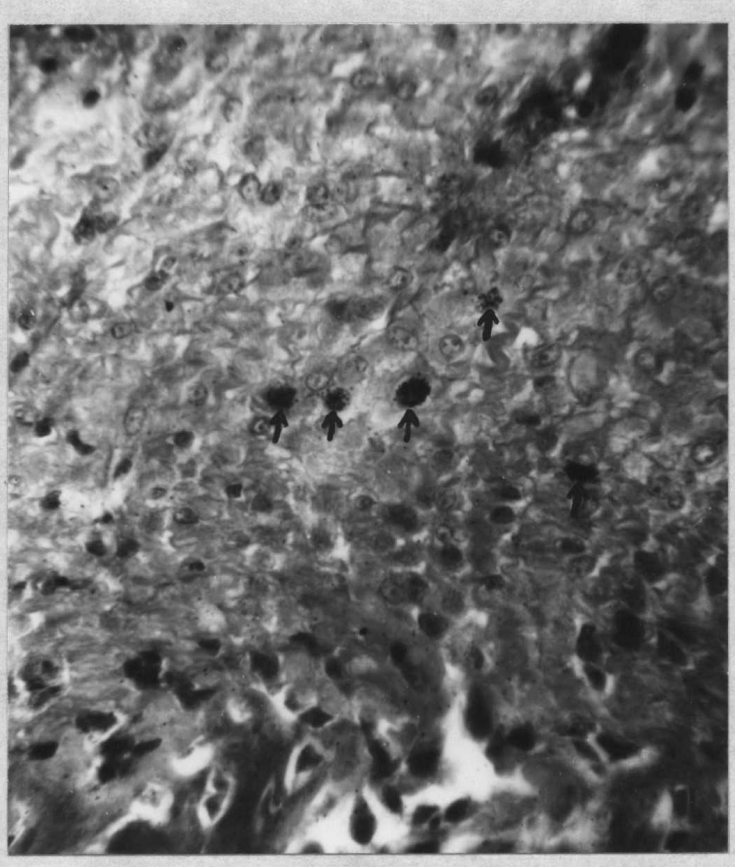

Figure 25

PLATE XIII

PLATE XIV

Figure 26. Photomicrograph of an autoradiograph of a group II 4-day developing exostosis. Note that the majority of labeled cells are outside the zone of intercellular material. P., proliferative zone; I.M. zone of intercellular material; N.B., zone of intramembranous bone formation. (Periodic Acid-Schiff and Hematoxylin) X101.

Figure 27. Photomicrograph of an autoradiograph of a 12-day developing exostosis. The section was taken distal to the center of insertion. Note that there are very few labeled cells in the proliferative zone in this section. P., proliferative zone; I.M., zone of intercellular material. N.B., zone of intramembranous bone formation. (Periodic Acid-Schiff and Hematoxylin) X101

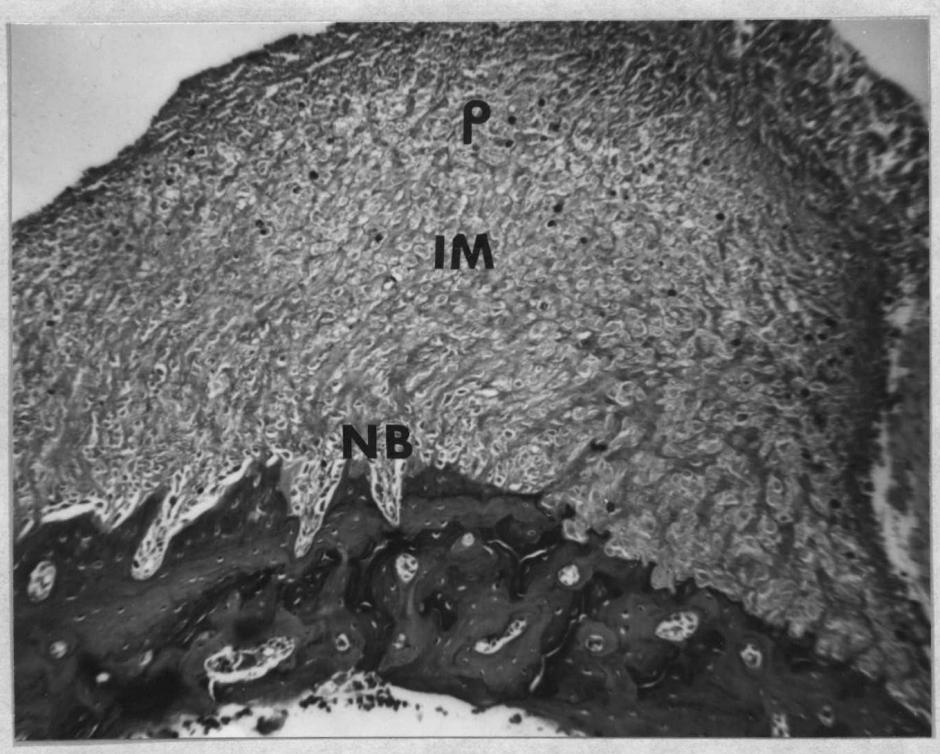

Figure 26

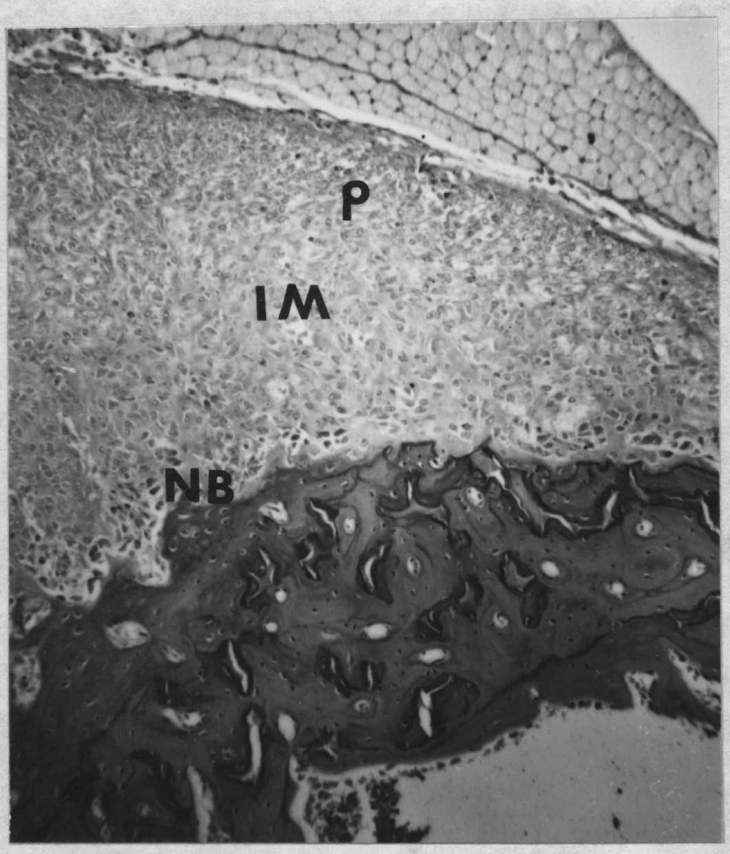

Figure 27

PLATE XIV

PLATE XV

Figure 28. Photomicrograph of an autoradiograph of a 12-day developing exostosis. The section was taken from near the center of adductor longus-pectineus insertion. Note the numerous labeled cells in the proliferative zone. p., proliferative zone; i.m., zone of intercellular material; n.b., zone of intramembranous bone formation. (Periodic Acid-Schiff and Hematoxylin) X101.

Figure 29. Photomicrograph of an autoradiograph of a 7-day developing exostosis of group II. This section was taken from near the center of adductor longus-pectineus insertion. Note the numerous labeled cells on the pectineus side of the exostosis. p., proliferative zone; i.m. zone of intercellular material; n.b., zone of intramembranous bone formation. (Periodic Acid-Schiff and Hematoxylin) X50.

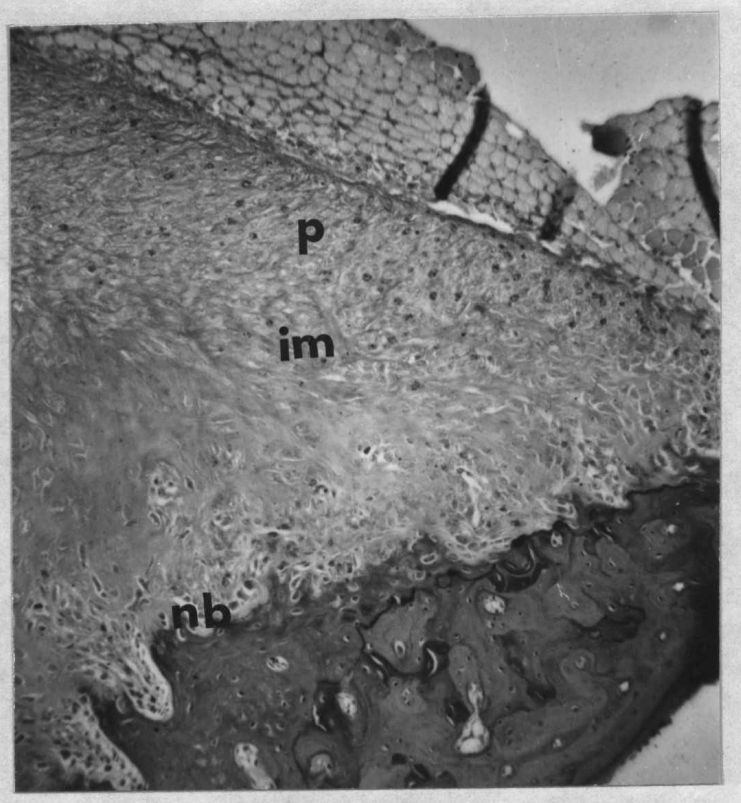

Figure 28

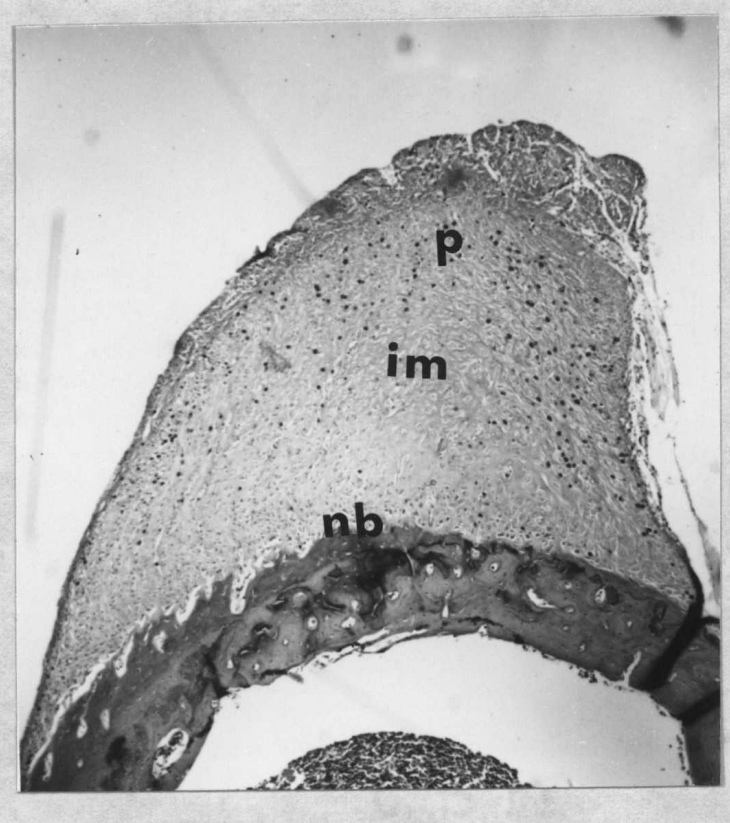

Figure 29

PLATE XV

PLATE XVI

Figure 30. Photomicrograph of an autoradiograph of a 7-day developing exostosis of group II. The dark line roughly seperates the proliferative zone from the zone of intercellular material. Note that most of the labeled cells in the zone of intercellular are located toward the outer edge of the zone. p., proliferative zone; i.m. zone of intercellular material; n.b. zone of intramembranous bone formation. (Periodic Acid-Schiff and Hematoxylin) X101.

Figure 31. Photomicrograph of an autoradiograph of labeled preosteoblasts and a labeled osteoblast in the zone of intramembranous bone formation of a group II 4-day developing exostosis. 1., labeled preosteoblasts; 2., labeled osteoblast. (Periodic Acid-Schiff and Hematoxylin) X420.

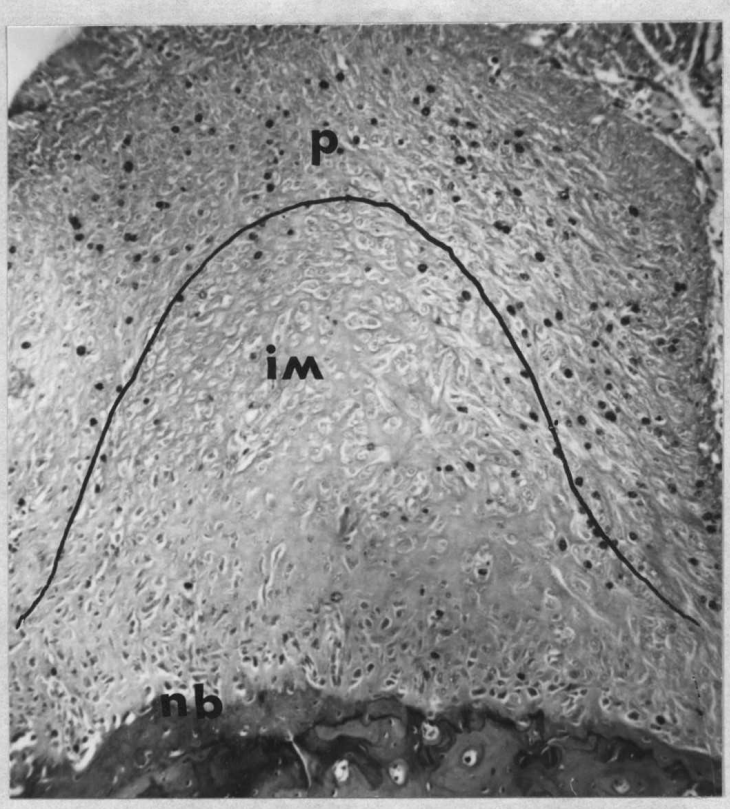

Figure 30

Figure 31

PLATE XVI

PLATE XVII

Figure 32. Photomicrograph of an autoradiograph of the osteogenic layer of periosteum from a group IIIa 2-day developing exostosis. Note the random distribution of labeled cells in all areas of the osteogenic periosteum. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 33. Photomicrograph of an autoradiograph of a group IIIa 4-day developing exostosis sacrificed 3 days after H^3TDR injection. Arrows indicate cells with heavy accumulations of silver grains over their nuclei in all three zones. (Periodic Acid-Schiff and Hematoxylin) X101.

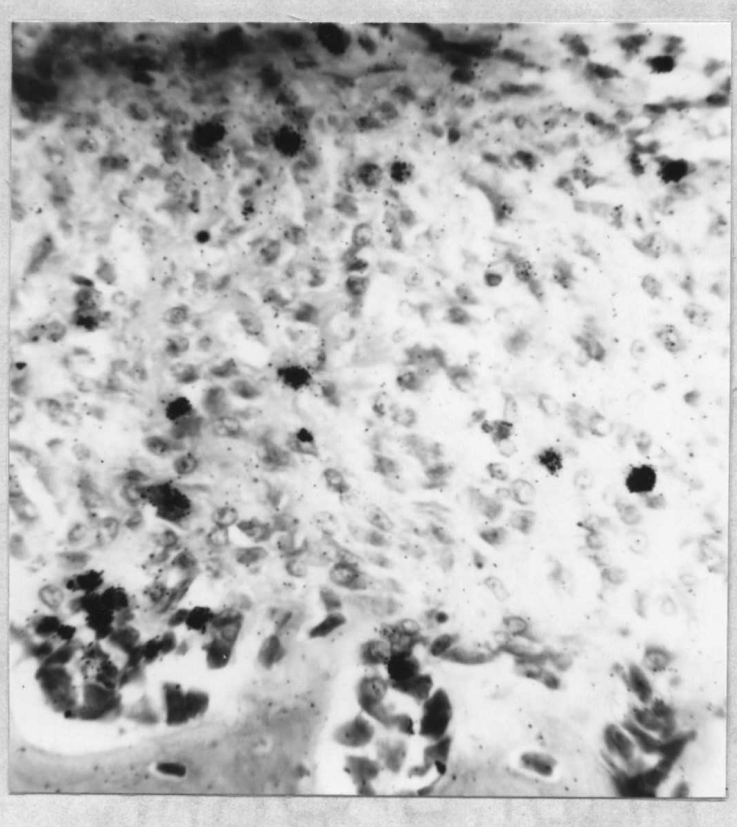

Figure 32

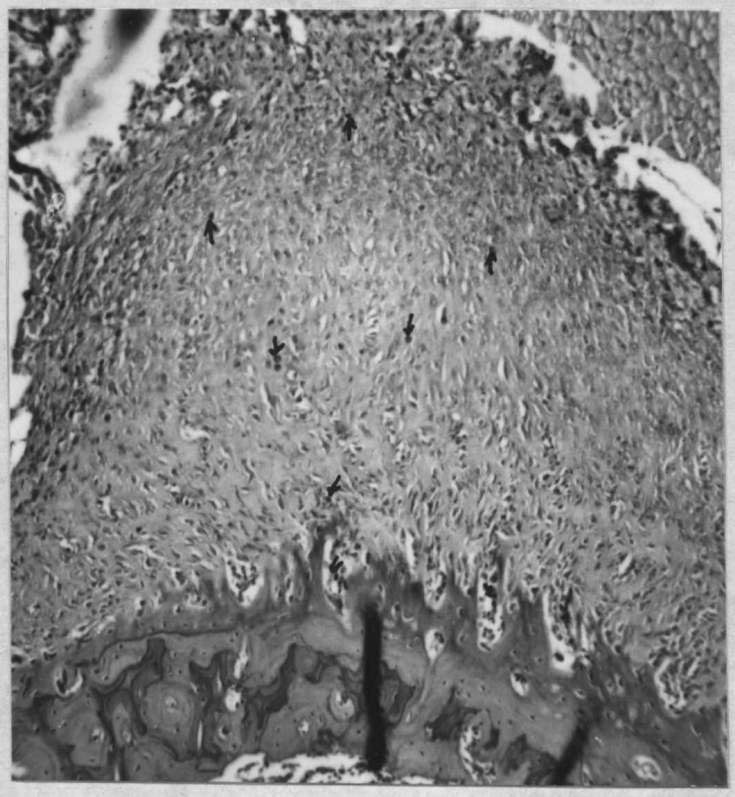

Figure 33

PLATE XVII

PLATE XVIII

Figure 34. Photomicrograph of an autoradiograph of the deeper zones of a developing exostosis from a 7-day lathyric rat that was sacrificed 6 days after H^3TDR injection. 1., labeled pre-osteoblast; 2., labeled osteoblast; 3., labeled osteocyte. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 35. Photomicrograph of an autoradiograph of a group IIIb 7-day developing exostosis that had been labeled with H^3TDR 4 days after the initial injection of BAPN. Arrows indicated labeled cells in all three zones of the developing exostosis. (Periodic Acid-Schiff and Hematoxylin) X101.

Figure 34

Figure 35

PLATE XVIII

PLATE XIX

Figure 36. Photomicrograph of an autoradiograph of the proliferative zone of a 9-day developing exostosis that was labeled with H^3TDR 4 days after the initial injection of BAPN. Note the abscence of heavily labeled cells. Arrows indicate some lightly labeled cells. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 37. Photomicrograph of an autoradiograph of the zone of intramembranous bone formation from the same animal as Figure 35. 1., labeled preosteoblast; 2., labeled osteoblast; 3., labeled osteocytes. (Periodic Acid-Schiff and Hematoxylin) X420.

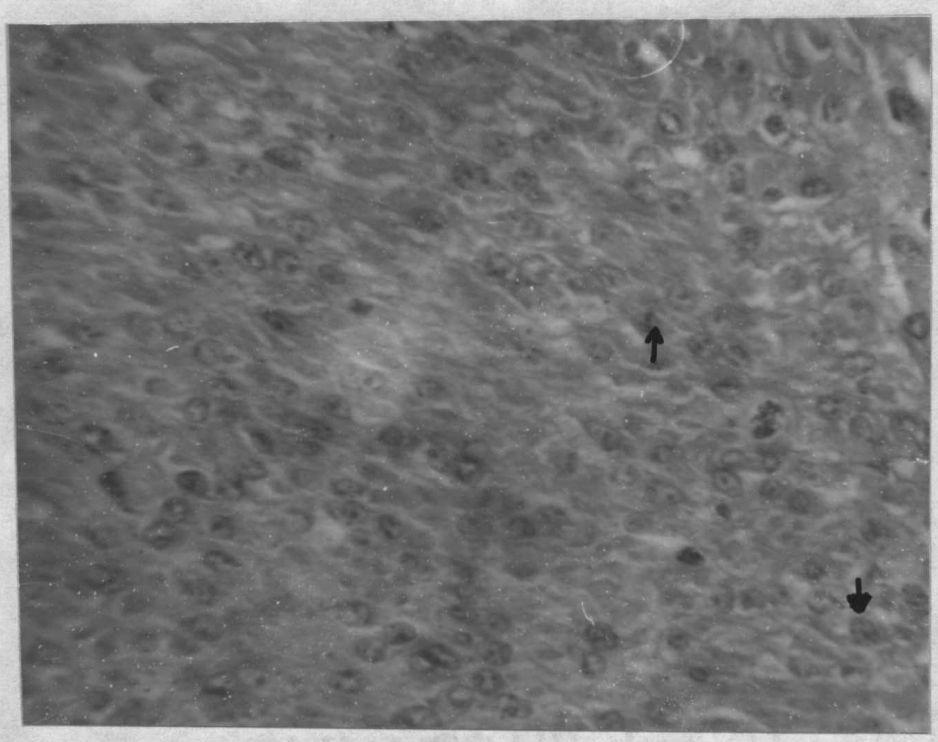

Figure 36

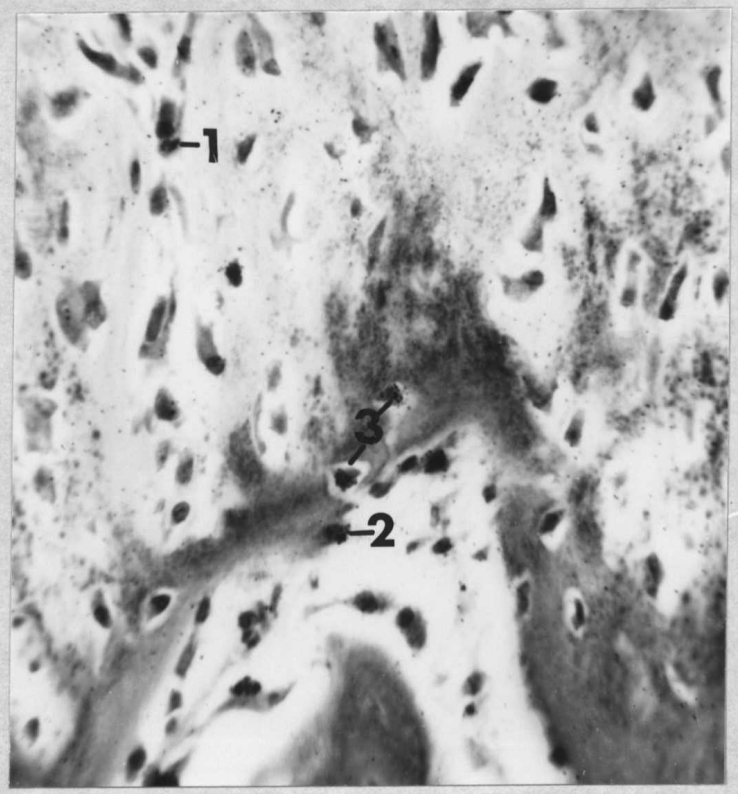

Figure 37

PLATE XIX

PLATE XX

Figure 38. Photomicrograph of the proximal epiphyseal plate of a 9-day lathyric rat showing slight disorganization of the epiphyseal plate. (Hematoxylin and Eosin) X101.

Figure 39. Photomicrograph of an autoradiograph of proximal tibial epiphyseal cartilage from a rat sacrificed 2 hours after H^3TDR injection. The labeled cells are localized to the zone of proliferating cartilage cells. (Periodic Acid-Schiff and Hematoxylin) X420.

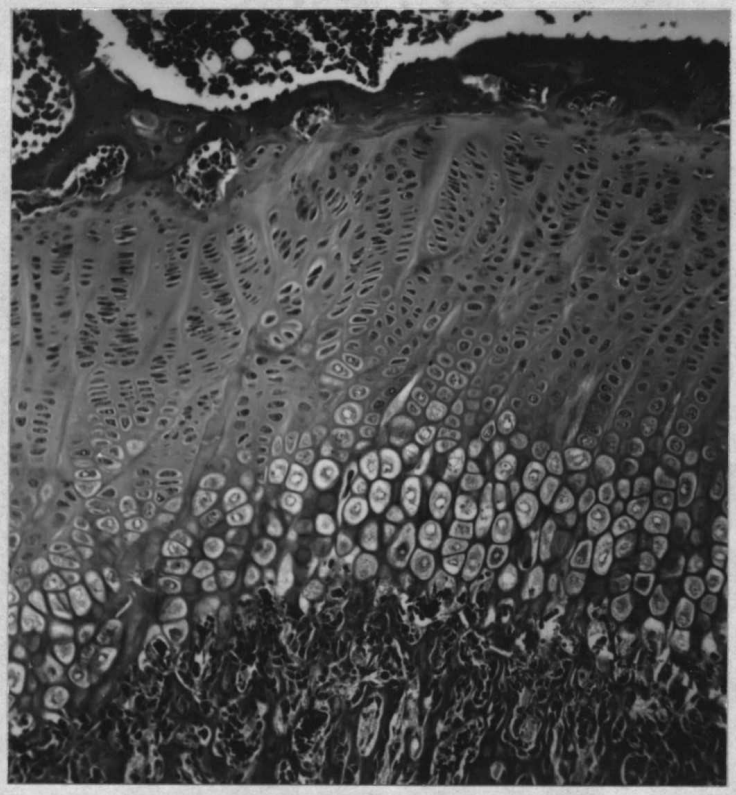

Figure 38

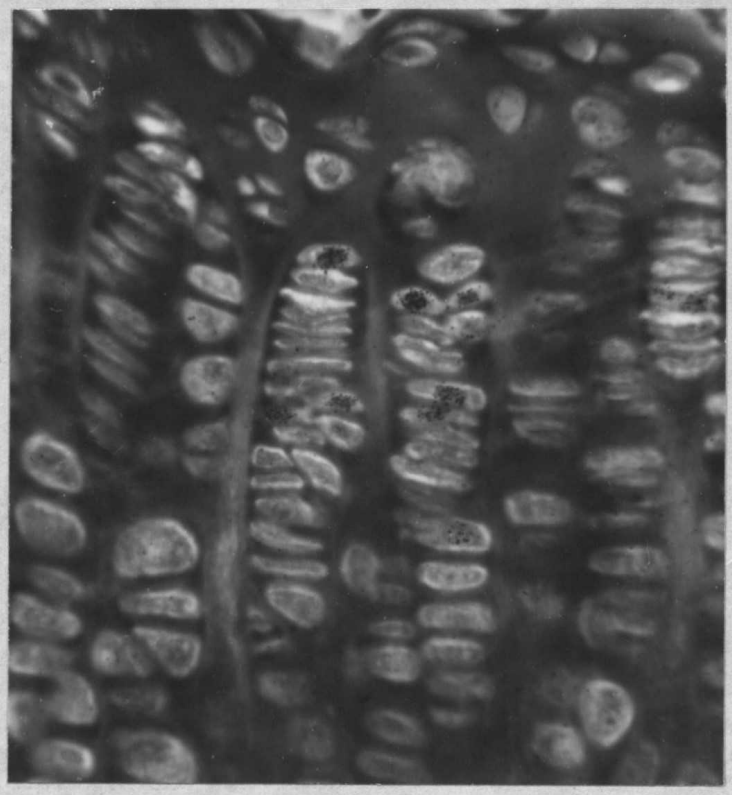

Figure 39

PLATE XX

PLATE XXI

Figure 40. Photomicrograph of an autoradiograph of proximal epiphyseal plate of the tibia from an animal sacrificed 16 hours after H^3TDR injection. Labeled cells are now present in the zone of maturing cartilage cells. (Periodic Acid-Schiff and Hematoxylin) X420.

Figure 41. Photomicrograph of an autoradiograph of adrenal cortex from a group II control rat. The arrows indicate labeled cells. c., capsula; g., glomerulosa; t., transition zone; f., outer fasciculata. (Periodic Acid-Schiff and Hematoxylin). X420.

Figure 40

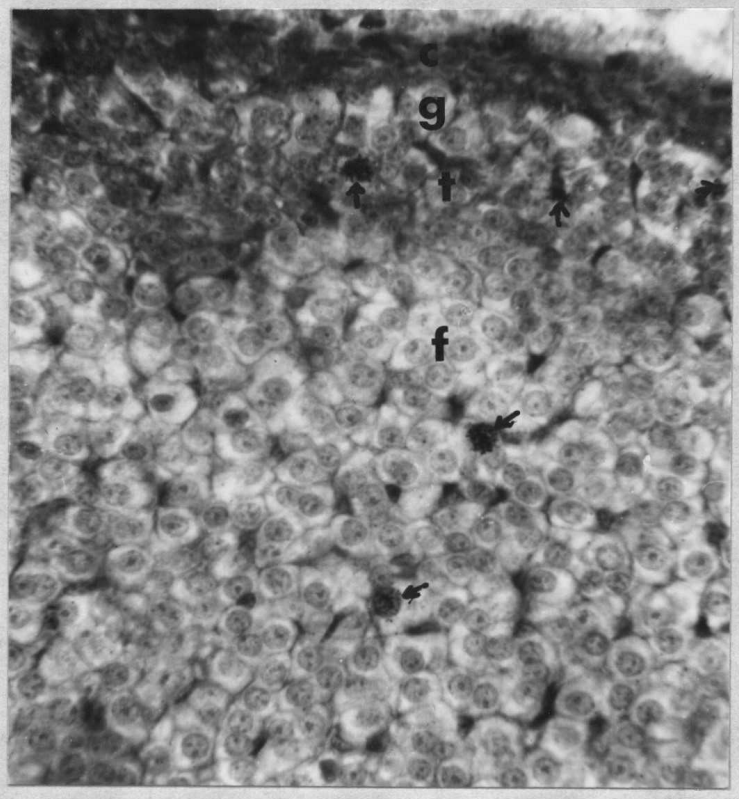

Figure 41

PLATE XXI