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Influence of Protein Diets on the Development of Abnormalities of the Aorta and Skeleton of Mature Lathyric Rats

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INFLUENCE OF PROTEIN DIETS ON THE DEVELOPMENT OF ABNORMALITIES OF THE AORTA AND SKELETON OF MATURE LATHYRIC RATS

by

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CHAPTER I

Introduction

The broad, general background of this study is the paralyzing, neurological disease of man associated with the consumption of large quantities of certain peas as an article of the diet. The disease known by the name of lathyrism is of uncommon occurrence at the present time but was of fairly common occurrence in the past. The historical background for this study is therefore the very extensive literature related to the nature and occurrence of the disease in man and attempts of investigators to produce the disease in experimental animals. Experimental studies of the disease have been numerous in recent years, particularly those which have employed the rat as the experimental animal. The recent historical background for this study is therefore the literature on lathyrism in the rat. The review of lathyrism to be presented below will be limited to the disease in man and the characteristics of the disease in the rat.

The paralyzing disease of man was not identified by name until 1873 when, according to German (1), Cantani called the disease lathyrism because peas of the genus Lathyrus appeared to produce the disease. Descriptions of the disease, or

similar diseases, according to Denny-Brown (2) and Selye (3), are found in the writings of Hippocrates, Pliny, Galen and Avicenna. These indicate that the disease occurred from early times in Europe and Africa. Singh and Singh (4) state that ancient Indian literature also includes descriptions of the disease thereby indicating that the disease also occurred in Asia. The disease therefore appears to have been of fairly widespread occurrence in ancient times.

Clinically, human lathyrism is a neurological disease with characteristic and consistent symptoms. It usually develops rapidly following exposure to cold or fatigue. A progressive weakness of the lower extremities accompanied by sensory disturbances such as pain, tingling or numbness signal the onset. Then spasticity and rigidity of the lower limbs with a loss of the disturbances of sensation become the prominent features of lathyrism causing the victim to walk in a characteristic manner on the balls of his feet with his knees slightly flexed (2, 5). Therefore, lathyrism is a spastic paraplegia indicative of damaged descending spinal cord tracts.

This disease is rarely fatal and thus the pathological characteristics have not been studied to any great extent. Only one report of the histopathological picture of the disease is recorded in the literature. The case examined was found to have degeneration and gliosis of the lateral column of the spinal cord on both sides and shrinkage of the Betz cells in the upper part of the anterior central gyrus. The sensory cells all

appeared normal (6).

Although most investigators feel that human lathyrism is caused by a toxin present in the Lathyrus peas the etiology of the disease has remained elusive. Dilling (7) extracted two alkaloids from Lathyrus sativus, one of the species implicated in lathyrism, and found they produced paralysis when injected into frogs. Stockman (8) stated the toxicity of Lathyrus peas was due to an acid salt of phytic acid because neurological symptoms could be produced in monkeys upon injection. Rudra (9) has implicated selenium as the toxin present in Lathyrus sativus, since these peas contain high levels of selenium which may interfere with the metabolism of methionine. Still other investigators suggested that the disease is not caused by the peas per se but is the result of a deficiency of one of the amino acids (10), a deficiency of a vitamin (11), or it is caused by toxic fungi or virus infection together with malnutrition (cited from 12). The Lathyrus peas used in making the meal are often contaminated with common vetch, Vicia sativa. The seeds of this plant were shown to contain an alkaloid which caused neurological symptoms (cited from 2). A neurotoxic amino acid, beta-cyanoalanine, has recently been isolated from Vicia sativa which was thought to be a possible cause of the symptoms seen in human lathyrism (13).

Efforts to produce in experimental animals a disease comparable to lathyrism in man have for the most part been unsuccessful. However, the search for a suitable experimental

animal for the study of lathyrism lead to the discovery of a new type of disease produced in the rat by feeding diets containing the sweet pea Lathyrus odoratus. Lathyrism in the rat is primarily characterized by the production of deformities of the skeleton and less frequently by aneurysms of the aorta. The literature related to these abnormalities and the work of identifying the agent in sweet peas which produce them will be reviewed below.

By feeding a meal made from the seeds of the common flowering sweet pea (Lathyrus odoratus) to rats, Geiger, Steenbock, and Parsons (14) found extreme skeletal deformities could be produced. No central nervous system damage was evident even after the young rats had consumed a 50 per cent sweet pea meal diet for long periods. Instead severe curvature of the spine, sternal curvature, malformations of long bones and hernias were produced by this diet.

Since the disease in rats produced by Lathyrus odoratus appeared to be a completely different entity from the one seen in man attempts have been made to amend or change the name of the disease produced in rats. Vivanco and Jiminez Diaz (15) suggested "Odoratism" would be a more appropriate term. Selye (3), in his comprehensive review, used the names "Neurolathyrism" and "Osteolathyrism" to distinguish between the two types of disease produced by the consumption of Lathyrus peas. Yet, the terms "Lathyrism" and "Experimental Lathyrism" are still commonly used as the name for the disease in rats.

Immediately upon the discovery of osteolathyrism, investigators tried to determine what constituent of the sweet pea was responsible for the production of the disease. It was found that the sweet peas would not produce the disease if fed to rats after extraction with cold water (14,16). Dupuy and Lee (17) isolated a crystalline substance from the Singletary pea which was effective in producing lathyrism in rats and in the same year Schilling (18) isolated a similar crystalline material from Lathyrus odoratus which also produced the disease in rats. The crystalline toxin from the Lathyrus peas was identified as beta- (N-gamma-L-glutamyl)-aminopropionitrile (19). Dasler (20) and Ponseti and coworkers (21), working independently, both recognized that the gamma-glutamyl moiety of the molecule was not essential and the beta-aminopropionitrile portion was the active component. Bachhuber, et al. (22) noted that beta-aminopropionitrile (BAPN) would become biologically inactive if a methyl or acetyl group were substituted into the amino group or if a hydroxy were substituted for the amino group. A number of compounds chemically closely related to beta-aminopropionitrile have been tested for their biological activity on experimental animals. Of those tested, amino-acetonitrile (AAN) (21) and beta-mercaptoethylamine (23) produced skeletal lesions similar to those caused by BAPN.

One of the most interesting and most practical aspects of experimental lathyrism in the rat is the development of aortic aneurysms. It is interesting from the standpoint of being the

only known way of producing dissecting aneurysms experimentally by a diet; and it is practical in that it is a method for studying dissecting aneurysms which develop in much the same manner as they develop in human beings with Marfan's Syndrome.

In 1952, Ponseti and Baird (24) demonstrated that weanling rats fed a 50 per cent Lathyrus odoratus diet spontaneously developed dissecting aortic aneurysms after 5 weeks on the diet. The aortas exhibiting aneurysms showed scattered areas of medial necrosis along the thoracic portion with extensive areas of "dissection" where the fibers of the media were split by blood entering from the lumen. Ponseti and Shepard (25) noted that the incidence of dissecting aneurysms of the aorta was related to the age when rats were started on the lathyrogenic diet. None of the rats started on the diet when 51 days old or older developed aneurysms, but 75 per cent of the weanling rats developed dissecting aneurysms. The lesion started in the media with no apparent disruption of elastic fibers but there appeared to be a loss of cohesion between the fibers related to loss of binding of the ground substance. No lesions were observed in the smaller arteries or veins.

A study by Bachhuber and Lalich (26, 27) showed that aneurysms in young rats were produced only when a 10 per cent casein diet was fed and no aneurysms developed when 20 per cent casein was employed. They found practically all dissecting aneurysms in the arch of the aorta. Microscopically, the affected aortas revealed degeneration and fragmentation of the elastic fibers together with medial edema leading to aneurysmal

dilitation or medial hemorrhage or both. Churchill, et al. (28) basing their findings on a histochemical study concluded the changes appeared to be associated with an increase in mucopolysaccharides and Pyörälä, et al. (29) stated there was an increase in histochemically demonstrable acid mucopolysaccharides in aortas from lathyrus-fed rats.

Whereas, Walker and Wirtschafter (30) stated that the histopathogenic basis of dissecting aneurysms in lathyric rats is elastinolysis followed by a fibroblastic proliferating reparative phase. In a later study, Walker (31) gave the lathyrogenic factor to rats from birth and found a decreased number of elastic fibers in a variety of tissues. From this he concluded the disturbance in the aorta caused by lathyrism involved the inhibition of normal elastic fiber formation rather than an elastolytic process. A disorganization of normal morphology and an increase in acid mucopolysaccharides in the ground substance especially along the elastic fibers was cited by Wirtschafter (32) as indicating a profound metabolic disruption in lathyric aortas. Wadja, Lehr, and Kruhowski (33) found no sex difference in the severity of the skeletal lesions of weanling rats but the overall incidence of aortic rupture in female animals was considerably lower than in males. They assumed a true hormonal difference was involved since androgens enhanced the severity and rate of medionecrosis of the aortas they studied.

A histological and histochemical study by Menzies and Mills (34)revealed 4 phases in the production of aortic aneurysms in young rats. The first phase was an increase in ground substance in the media as demonstrated by alcian blue or metachromatic toluidine blue staining. The second phase involved reticulin fiber disintegration which impaired the adhesion between elastic laminae leading to blood passage into the media which was the third phase. The fourth and final phase was that of repair. The elastic laminae in the area seemed to disappear and were replaced by fibroblasts and collagen. From their observations it was concluded that several factors are involved in experimental lathyrism. Degeneration of ground substance, interference with normal growth and fibrous proliferation are all involved.

A series of investigations much like the one of Menzies and Mills was undertaken by Gillman and Hathorn (35, 36, 37). They observed that the aortas in lathyric rats were most sensitive to the lathyrogenic factor when the aortas were growing at the greatest rate, that is, when the rats were under 28 days of age. They suggested that some disturbance in neogenesis of connective tissue fibers during growth and regeneration is caused by the lathyrogenic agent and thus aortic rupture occurs in the young rats. The failure seems to be in the ground substance at some stage after sulfation of the mucopolysaccharides. The aortic ruptures in weanling lathyric rats were probably due to a depressed synthesis of new collagen-like cores of vascular elastic membranes rather than lysis of pre-existing ones.

Lalich, Wirka, and Angevine (38) analyzed the aortas from BAPN treated young rats and found that collagen was synthesized during repair but there was nothing to suggest that elastin is being synthesized in repair of aortic aneurysms. An in vitro investigation done by McCallum (39) supported the hypothesis that aortic rupture in experimental lathyrism is secondary to the failure of normal elastic fiber formation.

An electron microscope study of the aortas from lathyric rats was done by Keech (40). The ultrastructure of the initial changes demonstrated an increased thickness of the wall of the aorta, a rearrangement of the smooth muscle cells from a normal oblique position to a radial orientation, and a progressive increase in fine electron dense material on the surface of elastic membranes. One adult lathyric rat aorta was studied with the electron microscope but no changes from normal were observed.

Recently, Zahor and Cyzbanova (41) studied the intrauterine effect of low doses of the lathrogenic factor on the aorta of embryos. If Lathyrus peas comprise 10 per cent of the pregnant rats' diet about 80 per cent of the living offspring developed fibroelastosis of the aorta. They concluded that the fibroelastosis is the result of healing of an aneurysm in the fetal aorta whereas healing of an aneurysm in a weanling rat is accomplished by collagenous tissue formation. Fetal rats may have the ability to form new elastic tissue while older animals are incapable of synthesizing new elastin.

The early investigations made on osteolathyrism were primarily concerned with the skeletal changes in young rats. Geiger, Steenbock and Parsons (14) noted that the rats developed kyphoscoliosis, sternal curvature, enlargement of costochondral junctions and malformation of long bones. Robinson and Bast (42) observed that exostoses of bone would appear in the regions of important muscle attachments. Ponseti and Shepard (25) described the effect of the lathyrogenic agent upon the epiphyseal plates of rapidly growing weanling rats.

Ponseti and Shepard noted a pronounced widening of the epiphyseal plates of long bones with an increase in number and size of cartilage cells and a loss of cohesion of the cartilage matrix. After 3 to 6 weeks on the lathyrogenic diet they reported a disintegration of the cartilage matrix between the zone of proliferating cells and the calcified cartilage. Ramamurti and Taylor (43) stated that the widening of the epiphyseal plate in young lathyric rats was due to an increase in the "zone of maturing cartilage cells" followed by a retardation of the later stages of endochondral ossification. The basic lesion was felt to be caused by an alteration in the ground substance of the epiphyseal cartilage. Autoradiographic studies done by Belanger (44) on the epiphyseal cartilage in lathyric rats using radioactive sulfate seemed to indicate that there was an equal or slightly higher sulfate fixation per individual cell in the cartilage from these animals as compared to normal rats, and since there were more cartilage cells in the epiphyseal plates from lathyric animals, the overall uptake

of sulfur by cartilage was apparently greater. But Karnovsky and Karnovsky (45) found the incorporation of radioactive sulfate into epiphyseal cartilage from rats was inhibited by lathyrogenic agents.

The first description of the exostoses of long bones in lathyrus-fed rats was that of Robinson and Bast (42). They described the exostoses of the femur from rats fed the Lathyrus pea diet for 4, 7, and 13 weeks. Histologically the bone growths appeared much like the callus formation of fracture repair exhibiting rapid proliferation of osteogenic tissue under the periosteum with formation of new bone spicules next to the old cortical bone. They suggested that perhaps the stimulus for exostosis formation was a spastic muscle pull at muscle attachment sites since these were the only areas affected.

Osteoporosis was the first microscopic change in long bones of weanling lathyric rats observed by Ponseti and Baird (24). Formation of new subperiosteal bone in the metaphyses of long bones and thickened cellular periosteum overlying islands of new connective tissue were also noted. Ponseti and Shepard (25) stated that the formation of new bone under the periosteum occurred first at the site of muscle attachments and appeared to follow the lifting of the periosteum by muscle pull.

Yeager and Hamre (46) described the early histological changes which take place in exostosis formation in adult lathyric rats. The first phase of exostosis formation was noted on the first day following lathyrus feeding and was characterized

by a rapid proliferation of the cellular inner layer of the periosteum. No signs of hemorrhage, edema, or injury could be detected at the muscle insertion which would suggest that the periosteum had become detached and initiated the proliferation. The fibroblastic proliferation proceeded rapidly for the first few days and not until about the seventh or eighth day of lathyrus feeding did osseous tissue begin to form. The newly formed bone and marrow spaces were formed within the fibrous connective tissue of the thickened periosteum by intramembranous osteogenesis. Outside of the newly formed bone toward the muscle, the outer zone of cells in the periosteum were seen to continue proliferating and increasing the size of the exostosis. The formation of exostoses at the sites of muscle attachments appear to be dependent upon the presence of tension of muscle and the lathrogenic agent. Sectioning of the muscle or denervating the muscle prevents the formation of an exostosis in a lathyrus-fed rat (47, 48).

Yeager and Gubler (49) described the appearance of the adductor longus insertion in a rat given BAPN for 7 days. The changes leading to exostosis formation were seen in the inner layer of the periosteum where 3 zones could be seen. The outer zone was the proliferative zone consisting of mesenchymal-like cells and very little connective tissue. The middle zone consisted of cells differentiating into premarrow areas and relatively acellular prebone areas. The inner zone, next to the cortical bone, was comprised of immature spicules of bone

separated by premarrow areas. Amato and Bombelli (50) noted the periosteum was raised in young lathyric rats. They suggested the raising of the periosteum may be due to extravasation of blood under the periosteum, loosening of Sharpey's fibers allowing the periosteum to be pulled away from the cortical bone, or due to muscle spasm. A study of the bone that was newly formed in lathyrus-induced exostoses was conducted by Berquist and Hulth (51). They noted that diffuse cellular proliferation in the deep periosteum was the initial change after lathyrogenic factor administration. After a week the exostosis started to develop radiating bone spicules. The vascular pattern was also radiating from periosteal vessels toward the vein in the medullary cavity. They stated that the manner of vascularization of the periosteum must have some influence upon the exostosis formation.

Several references to the role of protein in altering the course of experimental lathyrism have been made in the literature. Dasler (52) tested the effect of feeding a lathyrogenic diet high in hydroxyproline to young rats. This experiment was based on the fact that the metabolism of collagen, which is uniquely high in hydroxyproline, is disturbed in experimental lathyrism. The source of hydroxyproline used was gelatin, and casein served as the control protein. A group of weanling rats was also fed the sweet pea diet without excess protein. He found both gelatin and casein offered partial protection against the skeletal deformities, but they did not completely prevent

osteolathyrism. Casein was more effective than gelatin in delaying the onset of lathyrism perhaps because casein is a complete protein not deficient in essential amino acids. No explanation for the partial protective action of high levels of dietary protein was given. Bachhuber and Lalich (26) observed that no dissecting aneurysms were produced in young lathyric rats when they consumed a diet containing 20 per cent casein, but aneurysms appeared when a 10 per cent casein diet was fed.

Lee, Dupuy and Rolfo (53) conducted a study in which they maintained weanling rats on a diet containing Singletary pea meal and various concentrations of several types of protein. It was noted that when casein, lactalbumin, or gelatin comprised 20 or 25 per cent of the diet the effects of the lathyrogenic factor upon growth rate and skeletal changes were minimal, whereas the protein zein, in the same concentrations, had no effect. Since the protein of the Singletary pea is deficient in methionine they supplimented the diet with methionine. But adding methionine alone did not decrease the severity of the lathyrism. It was suggested that the depression of skeletal lesions in lathyrism by added protein may be the result of a reversal of interference with amino acid metabolism. The protein may act by providing a specific group of amino acids or a specific peptide.

Yet, Ponseti, et al. (54) could not demonstrate any protection against the skeletal lesions when a high protein

lathyrogenic diet was fed to weanling rats. The diets used consisted of 27.3 per cent casein incorporated into either a 35 per cent or 50 per cent Lathyrus pea meal diet. Likewise, Warram, et al. (55) found that excess dietary protein had no protective effect against osteolathyrism in the rat. They fed diets containing no protein, 27 per cent casein, or 64 per cent casein in addition to the BAPN and examined the epiphyseal plates from the various groups of rats to determine the severity of the lathyrism. They noted the skeletal deformities were greater in the animals receiving no protein but were the same for normal and high protein fed rats.

A histological study of the alveolar bone of weanling rats fed a diet consisting of 30 per cent ground sweet peas and various levels of added protein was carried out by Gardner (56). The alveolar bone of the young animals fed an unsupplimented sweet pea diet exhibited a marked increase in periosteal and endosteal osteoclastic activity and a decrease in normal bone apposition. This resulted in pronounced osteoporosis of the alveolar bone. But casein or gelatin supplimented lathyrogenic diets produced normal apposition of alveolar bone and no osteoporosis.

When BAPN was incorporated into various diets fed to young turkeys, dissecting aneurysms were produced. From this study it would appear that the source of dietary protein was a factor in aneurysm production since it was found that BAPN-induced angiorrhexis occurred faster and more frequently if the diet

contained fish meal protein (57).

Other investigations into the effects of dietary protein on the incidence of BAPN induced aneurysms have been done on rats (58, 59). Feeding Purina rat pellets and BAPN to young rats resulted in aneurysm formation before skeletal lesions developed. However, feeding an 18 per cent or 24 per cent casein diet to rats receiving BAPN protected them against angiorrhexis.

Thus it would appear that the amount and type of protein in the diet of animals subjected to the lathyrogenic factor markedly affect the course of the disease. The purpose of this problem was to determine what gross and histologically demonstrable effects a low protein diet would have on adult lathyric rats. The aorta was studied because of the marked effect of dietary protein concentration on the development of dissecting aneurysms in weanling lathyric rats. It was thought that perhaps a low protein diet would also induce aneurysm formation in adult rats receiving BAPN. The exostosis which forms at the pectineus-adductor longus insertion site of the femur was selected for study as an index of the severity of skeletal response to BAPN in adult rats. This exostosis was selected because Yeager and Hamre (46) have shown that this is one of the first areas of the skeleton of adult rats to be affected by the lathyrogenic agent.

CHAPTER II

MATERIALS AND METHODS

Two diets were used in the experiment, one considered to be a low protein diet and the other a diet with an adequate protein content. Animals which received an adequate protein diet were fed a 27 per cent casein diet* and rats placed on a low protein diet were fed an 8 per cent casein diet*. The composition of the diets employed appears in Table 1.

TABLE 1. - Composition of test diets.

Normal Protein Test Diet GBI

Ingredients	Composition
Vitamin-Free Test Casein GBI	27.0%
Starch	56.0
Vegetable Oil (hydrogenated)	14.0
Salt Mix, U.S.P. XIV	3.0
Vitamin Supplements	**Gms/100 lbs.**
Alpha Tocopherol	10.215
Calcium Pantothenate	2.043
Carotene in Oil GBI Type 39	67.000
Choline Chloride	272.400
i-Inositol	13.620
Menadione	.102
Niacin	27.240
Pyridoxine HCl	.953
Riboflavin	.953
Thiamine HCl	.953
Viosterol 400,000 U/gm.	3.000

*obtained from General Biochemicals, Chagrin Falls, Ohio.

Protein Deficient Test Diet GBI

Ingredients	Composition
Vitamin-Free Test Casein GBI	8.0%
Starch	75.0
Vegetable Oil (hydrogenated)	14.0
Salt Mix, U.S.P. XIV	3.0

Vitamin Supplements	Gms/100 lbs.
Alpha Tocopherol	10.215
Calcium Pantothenate	2.043
Carotene in Oil GBI Type 39	67.000
Choline Chloride	272.400
i-Inositol	13.620
Menadione	.102
Niacin	27.240
Pyridoxine HCl	.953
Riboflavin	.953
Thiamine HCl	.953
Viosterol 400,000 U/gm.	3.000

The lathyrogenic factor was administered to the experimental animals by placing beta-aminopropionitrile fumarate* in the drinking water. It was given at the rate of 150 milligrams of BAPN per 100 milliliters of water. A measured amount of water containing BAPN was given daily to each experimental animal and the amount that each rat drank was measured and recorded.

The 78 animals used in this study were adult female albino rats obtained from the Holtzman Rat Company, Madison, Wisconsin. Only females were used because males were not available at the time of the study. They were all 60 days of age and had an average body weight of 172 grams with a range of 152 to 194 grams. All of the animals were caged separately and each rat

*generously provided by Abbott Laboratories, North Chicago, Illinois.

was inspected and weighed daily.

The animals of this experiment were divided into 6 groups and the animals of each group subjected to the experimental regimen shown in Table 2 and described below.

Group 1 consisted of 12 rats which were given a normal protein diet plus water. The 12 animals of Group 2 received a normal protein diet and drinking water containing BAPN. Group 3 was comprised of 12 rats maintained on a low protein diet plus drinking water and the 12 animals of Group 4 received a low protein diet and BAPN in the drinking water. The rats in Groups 5 and 6 were started on the low protein diet at the same time as the first 4 groups and were maintained on this diet for 4 weeks before starting on the lathyrogenic agent. Group 5 consisted of 12 rats which remained on the low protein diet and water until they were sacrificed. This group of animals served as a control group for the 18 rats of Group 6 which were started on BAPN after being on the low protein diet for the first 4 weeks of the experiment.

Two rats from each of the first 4 groups were killed weekly starting at the end of the first week through the sixth week. Group 5 animals were killed weekly beginning with the end of the fifth week at the rate of 2 animals per week through the tenth week. The rats in Group 6 were killed at the end of week 5 and weekly through week 10. Two rats were killed on weeks 5, 6, and 7, and 4 animals were taken weekly for the remainder of the experiment.

TABLE 2. - The groups of rats on the experiment.

Group	Number of animals	Management of animals	Length of time on experiment
1	12	27% protein diet; drinking water	2 animals killed per week
2	12	27% protein diet; water containing BAPN	2 animals killed per week
3	12	8% protein diet; drinking water	2 animals killed per week
4	12	8% protein diet; water containing BAPN	2 animals killed per week
5	12	8% protein diet; drinking water	2 animals killed per week
6	18	8% protein diet; water for 4 weeks, then water containing BAPN	2 animals killed per week for first 3 weeks, then 4 animals killed per week.

All animals were killed with ether anesthesia and immediately skinned. The thoracic cavity was opened and the heart, aortic arch and thoracic aorta were removed and placed in fixative. All of the muscles surrounding and attached to the right femur with the exception of the pectineus and adductor longus were removed to expose the entire bone. The femur together with the attached pectineus and adductor longus muscles were removed and emersed in fixative. The viscera were examined and the remainder of the carcass was preserved in a 10 per cent formalin solution.

The ascending aorta and aortic arch were fixed in 10 per cent formalin and the descending thoracic aorta was fixed in

Zenker-formal (Helly's) solution. The different portions of the aorta were separately dehydrated, embedded in Tissuemat and sectioned serially at 7 micra.

The portions of the aorta fixed in 10 per cent formalin were stained with 1 of 5 stains. Harris' hematoxylin and eosin were employed for the demonstration of general morphology. Masson's trichrome stain was used to reveal the connective tissue components and Gomori's aldehyde fuchsin method demonstrated the elastic fibers. Periodic acid-Schiff (PAS) method for neutral mucopolysaccharides was used with a hematoxylin counterstain. The alcian blue stain was used to show acid mucopolysaccharides.

Sections of the descending thoracic aorta fixed in Helly's solution were stained by 4 different methods. Harris' hematoxylin and eosin stain and Masson's trichrome stain were also done on these sections. A combined alcian blue-periodic acid-Schiff stain was done to study the mucopolysaccharide distribution and a hematoxylin-eosin-azure II stain was run on the sections of the aorta for studying the cellular morphology.

The femurs were prepared for histological study in the following manner. Each right femur was fixed in Bouin-Hollande fixative (cupric acetate, 2.5 grams; picric acid, 4 grams, 40% formaldehyde solution, 10 milliliters; 2% trichloroacetic acid, 1.5 milliliters; distilled water, 100 milliliters). Each left femur was removed from the carcass which had been preserved in 10 per cent formalin and placed in fresh 10 per cent formalin

solution for one day. The femurs, both right and left, were decalcified in 10 per cent formic acid for 1 day. Each femur was then cut with a razor blade so that a block of tissue composed of approximately the middle third of the femur with the insertion of the pectineus and adductor longus was obtained for embedding. The trimmed piece of femur and attached muscles were decalcified 1 more day in 10 per cent formic acid and then washed for 1 or 2 days in running tap water. The tissue was dehydrated, embedded in Tissuemat in such a way that the femur would be cut in cross-section. Sectioning was done at 7 to 10 micra.

Each block of femur and muscle was sectioned entirely. For the femurs fixed in Bouin-Hollande solution, representative sections were selected at intervals of approximately 30 sections. The selected sections were placed on separate slides so different staining procedure could be employed on adjacent sections. The slides thus prepared were stained with Masson's trichrome stain or Harris' hematoxylin and eosin. One slide from each femur was stained with periodic acid-Schiff-hematoxylin.

Selected sections from femurs fixed in 10 per cent formalin were mounted on slides and stained with Masson's trichrome method. For reasons noted below, a few slides of particular femurs were stained with the metachromatic dye, Azure A.

The slides of both the aortas and femurs were studied with the light microscope.

Since the lathyrogenic agent causes enlargement of the adductor longus-pectineus insertion site a special method was used to determine the relative sizes of these areas. The largest cross-sectional area of this part of the femur was selected and projected into single weight photographic paper with a microprojector. The outline of the insertion was traced on the paper at the same magnification for all the femurs. The tracing was then cut out and weighed on a spring balance.*

* Roller-Smith, Bethlehem, Pennsylvania.

CHAPTER III

OBSERVATIONS AND RESULTS

The observations and results of this study will be presented under the following headings: (1) General Observations, (2) The Appearance of The Aorta of Control and Experimental Animals, (3) The Adductor Long-Pectineus Insertion of Control Animals, (4) The Exostoses of Lathyric Rats Fed Normal Protein Diet, (5) The Exostoses of Lathyric Rats Fed Low Protein Diet, (6) The Comparison of Exostosis Size of Normal Protein-Fed and Low Protein-Fed Lathyric Rats.

General Observations

The rats of this study were kept in individual cages. Each day for the period of the experiment, and a few days preceeding the experiment, each animal was examined for abnormalities and observed for behavior and the nature of movements and locomotion or other signs that could be a result of an experimental procedure.

All rats, except for a small number which exhibited a tendency to sneeze and sniffle, appeared in normal condition and health at the beginning of the study. The sneezing condition disappeared during the experiment and at the end of the

study all animals appeared healthy. No animal showed a discolored and rough fur coat, including animals fed a low protein diet and BAPN for several weeks. Animals of all groups were active. Possibly the most active and energetic animals were the animals of Group 6, fed a low protein diet and BAPN. The various procedures of the experiment did not appear to alter the behavior, the general appearance or health of the animals.

The animals of all groups were submitted to daily gross examination for evidences of abnormalities of the skeleton. None of the animals developed scoliosis or depressed thorax. Those abnormalities failed to appear in animals of this experiment. However, some of the animals of Group 2, fed the high protein diet and BAPN for the longer periods of time, exhibited to some degree the awkward gait associated with the presence of exostoses on the femur.

In an effort to obtain evidence of the influence of the low protein diet and BAPN on animals of this study, each animal was weighed daily and the average weight determined for each group of animals. The weekly average change of weight for each group of animals of this study is presented in Table 3.

The amount of weight gained by the animals in each of the different groups is shown in Figure 1. It can be seen that animals of Groups 3, 4, 5 and 6 fed the low protein diet increased in weight more slowly than animals of Groups 1 and 2

fed the normal protein diet. The animals on the normal protein diet and BAPN gained weight at a slightly slower rate than the normal protein-fed animals not given BAPN. However, administration of BAPN did not appear to influence the gain in weight of animals on the low protein diet.

TABLE 3. - Average body weights of animals of experiment.

Time in Weeks	Number of Animals	1	2	3	4	5	6
Beginning of Experiment	12	168	160	177	181	200	208
Week 1	12	193	189	179	181	204	210
Week 2	10	203	184	186	186	206	210
Week 3	8	216	221	198	196	219	220
Week 4	6	222	230	208	202	224	228
Week 5	4	234	232	210	210	221	231
Week 6	2	227	234	202	225	235	231

In this experiment it appeared important to know the amount of BAPN consumed by each animal and group of animals which received that agent. The average consumption of BAPN in milligrams is summarized in Table 4.

TABLE 4. - Average total consumption of beta-aminopropionitrile.

Number of Weeks on BAPN	Number of Animals in Each Group	Average BAPN Consumption in Milligrams per Animal for Each Week		
		Group 2	Group 4	Group 6
1	12	286	318	268
2	10	606	590	645
3	8	996	945	956
4	6	1370	1260	1245
5	4	1685	1600	1510
6	2	2090	1926	1730
Average Daily BAPN Consumption in Milligrams per Animal		47.5	45.3	43.0

The table above shows that animals of Group 2, fed the normal protein diet, drank more water per day and thereby received a higher dosage of BAPN than the animals of Groups 4 and 6, fed the low protein diet. The amount of BAPN consumed per gram of body weight for the animals of the various groups was determined and it was found that on this basis, there was very little difference between the groups. The animals of Group 4 were found to have received the most BAPN per body weight. The measurement of the quantity of water and BAPN was made to determine whether the quantity of BAPN consumed determined the size of the adductor longus-pectineus exostoses formed by the animals. It is doubtful that the differences in BAPN consumed is significant because some animals were observed to waste water in the process of drinking

and in other cases the water bottles would continue to discharge drops of water after the animals had stopped drinking. Furthermore when the size of the exostoses of individual animals was compared to the total BAPN consumed, a relationship was not found.

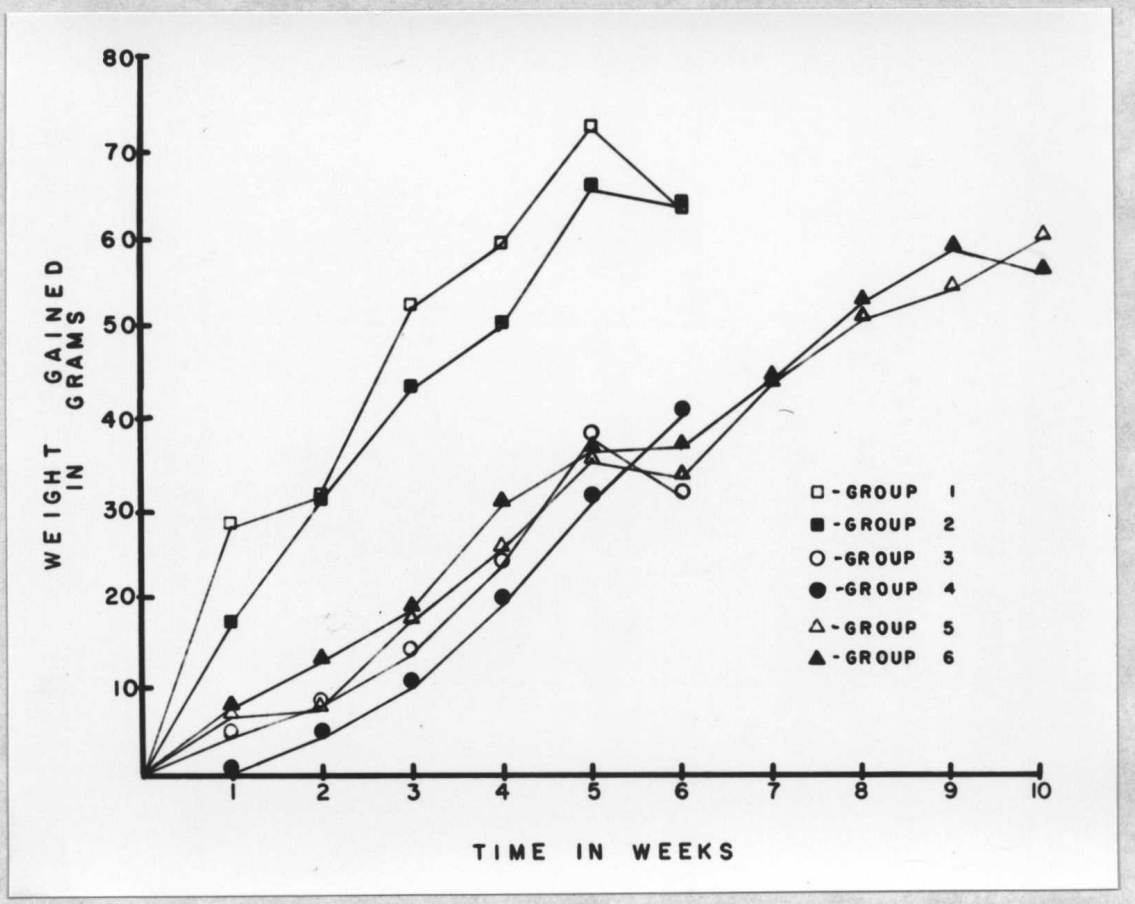

FIGURE 1. - Weight gain curve for each of the groups of rats in the experiment. Group 1, normal protein diet; Group 2, normal protein diet plus BAPN; Group 3, low protein diet; Group 4, low protein diet plus BAPN; Group 5, low protein diet for 10 weeks; Group 6, low protein diet for 4 weeks; then low protein diet plus BAPN.

All animals of this study were subjected to a complete autopsy examination for the presence of abnormalities that

could complicate the experiment. One animal of Group 5 was found to have a large subcutaneous, light gray colored encapsulated tumor located immediately anterior to the inguinal ligament of the right side. Seventeen of the 78 rats of the experiment possessed abscesses of the lung in various stages of development from small irregularly defined areas to large well-defined abscesses. Of the animals which possessed abscesses of the lungs, five animals were in Group 1, two animals in Group 2, two animals in Group 3, four animals in Group 4, two animals in Group 5 and two animals in Group 6. None of the animals killed after 6 weeks on the experiment possessed abscesses of the lung. Comparison of weight gain of animals which possessed or did not possess lesions of the lung showed no consistent differences. A comparison of the size of the adductor longus-pectineus exostoses in rats with lung disease to those of apparently healthy animals revealed that in a single instance the exostoses from a Group 2 rat with lung abscesses were considerably smaller than those found in the animals of that group which did not have abnormal lungs. No other correlation between exostosis size and the degree of lung disease was found. It appears that the pathological conditions observed did not influence the outcome of the experiment.

The skeletons of all animals were examined for the presence of exostoses or other abnormalities. The animals fed BAPN and either the low or high protein diet possessed exostoses at the

adductor longus-pectineus insertion of variable size and stage of development. None of the animals exhibited distortions of the spinal column, rib cage or sternum.

The Appearance of the Aorta of Control and Experimental Animals

Histological sections of the aorta of the rat showed it to be an elastic artery comparable in structure to the smaller elastic arteries of man. A tunica intima, tunica media and tunica adventitia were present but the tunica intima was very narrow and the tunica adventitia also fairly narrow when seen in cross-section.

In histological preparations the tunica intima varied in thickness with the endothelium, in some areas appearing to border directly on the internal elastic membrane. In other areas the endothelium and the internal elastic membrane were separated by a narrow space in which were found rows of fibroblasts. The endothelial cells possessed oval or round nuclei and the general shape of the central part of the cell was a rounded prominence which projected into the lumen. The cytoplasm of the endothelial cells extended as thin lines from the cell body and in many places appeared to border directly on the internal elastic membrane (Figures 3 and 4).

The tunica media was the widest and most prominent of the layers of the aorta of the rat. It was composed largely of alternating layers of elastic membranes and smooth muscle cells.

In cross-sections of the aorta the elastic membranes formed thick wavy bands that frequently branched and because they were fenestrated they occassionally appeared to be interrupted. The descending thoracic aorta was found to possess 7 to 11 elastic laminae while the ascending aorta was found to possess as many as 22 elastic laminae. In all cases the first 3 or 4 innermost elastic laminae appeared to be thicker than elastic laminae on the adventitial side of the vessel (Figure 5). The elastic membranes were found to stain dark violet with aldehyde-fuchsin, pink with eosin, deep red with the periodic acid-Schiff stain, and were unstained or stained very lightly with Masson's trichrome stain.

The areas of the tunica media of the aorta located between the elastic laminae was occupied by closely packed muscle cells. The muscle cells within an inter-laminar space were oriented in the same direction, chiefly in an oblique or longitudinal direction (Figure 6). Extending between the elastic laminae and thereby surrounding the muscle cells were fine elastic and reticular fibers (Figure 7). In PAS-hematoxylin stained sections reticular fibers were found to extend from one lamina to the next and were found to enclose individual muscle cells (Figure 8). Alcian blue staining demonstrated a green-colored amorphous substance to be present between the elastic membranes and the muscles of the inter-laminar spaces (Figure 9).

The tunica adventitia of the thoracic aorta of the rat was found to be sharply separated from surrounding periaortic fat (Figure 10) and to be composed of thick collagen fibers,

fibrocytes and vasa vasorum. The collagen fibers are arranged longitudinally throughout the adventitia but the fibrocytes appeared to have no regular arrangement. The vasa vasorum were present in sections of all levels of the aorta (Figure 11).

The histological pattern of the rat aorta appeared remarkably uniform. There were only two areas where the deviations from the above description occurred. One was in places where arteries were seen branching from the aorta and the other was the site of the attachment of the ligamentum arteriosum.

In sections taken through the aorta and one of the branches the elastic laminae of the media showed rearrangement and discontinuity. Some of the laminae appeared to run out into the arterial branch while others appeared interrupted at the point of branching (Figure 12).

The aorta was seen to have been altered at the point where the ligamentum arteriosum attaches. The ligamentum arteriosum in the rat appeared as a strand of connective tissue between the pulmonary artery and the aorta running in close proximity to the recurrent laryngeal nerve (Figure 13). The wall of the aorta where the ligamentum arteriosum attaches was composed mainly of collagenous connective tissue (Figure 14). At this point the tunica media exhibited interruption of the usual regular elastic laminae in its outer half. The laminae were replaced by irregularly arranged collagen fibers with fibroblasts interspersed between them (Figure 15).

The description given for the histological appearance of the aorta applies for all of the animals examined in this study. Pathological changes such as increased amounts of ground substance, elastic membrane disintegration, or hemorrhage into the tunica media were not observed in any of the aortas whether they were from low protein fed lathyric rats, normal protein fed rats, or normal rats.

The Adductor Longus-Pectineus Insertion of Control Animals

In the rat, the pectineus originates from the pubis ventral to the acetabulum and passes inferiorly and laterally to insert on the posteriomedial side of the linea aspera of the femur. The muscle has a rather long insertion extending from the base of the lesser trochanter to a point approximately two-thirds of the way down the shaft of the femur. The pectineus fans out from a rather narrow origin to a broad insertion. Thus, the lateral fibers are short and medial fibers are long.

The adductor longus takes its origin from a line along the ventral portion of the pubis. Its fibers pass inferiorly and laterally and converge into a thin flat tendon which inserts immediately posterior to the distal portion of the pectineus insertion. The tendon of the adductor longus inserts into the femur so close to the distal pectineus insertion that the two muscles have practically a common insertion. It is at this point on the femur that the pectineus-adductor longus exostosis is formed in experimental lathyrism in the

rat (Figure 16).

Histologically the periosteum at the pectineus-adductor longus insertion appeared different from the periosteum covering the femur where no muscles were attached. Generally, the periosteum of the rat femur was seen as a thin fibrous outer layer and thin inner cellular layer much like the descriptions of periosteum given in histology texts (Figure 17). But the periosteum in the region of the pectineus and adductor longus was markedly thickened (Figure 18).

The outer layer of the periosteum in the area of insertion of the adductor longus and pectineus muscles was observed to be only slightly thicker than that in other areas on the femur. It was composed of thick collagen fibers and elastic fibers with rather small spindle-shaped fibroblasts interspersed between the fibers (Figure 19). In cross-sectional areas taken through the adductor longus tendon the outer periosteal layer was reflected onto and blended with the surface of the tendon (Figure 20).

The inner layer of periosteum was much thicker in the region of insertion of the adductor longus and pectineus muscles than it was on other areas of this level of the femoral shaft. It appeared as a fairly cellular connective tissue layer overlying the cortical bone. The connective tissue was composed chiefly of collagen fibers from the tendon oriented in the direction of the pull of the muscles and periosteal cells lying between the collagen fibers. The

periosteal cells of the inner layer, in appearance resembled the fibroblasts in the outer layer, but were considerably larger and often exhibited a slightly basophilic cytoplasm. The nuclei of the periosteal cells were much larger and more rounded than the nuclei of the fibroblasts in the outer layer (Figure 21) and usually possessed two prominent nucleoli. Along the cortical bone of the femoral shaft an interrupted row of osteoblasts could be seen. They were recognized as osteoblasts by their intimate contact with the bone, by their irregular cuboidal shape, and by their darkly basophilic cytoplasm (Figure 22). Small blood vessels within the inner layer of the periosteum were also a prominent feature of the pectineus-adductor longus insertion site. The blood vessels were placed between and parallel to the bundles of collagenic fibers of the periosteum.

The Exostoses of Lathyric Rats Fed Normal Protein Diet

A marked enlargement of adductor longus-pectineus insertion was evident after 1 week on the lathyrogenic agent (Figure 23). In cross-section taken through the adductor longus-pectineus insertion, the inner layer of the periosteum at the insertion was seen to be greatly thickened; but the outer fibrous layer of periosteum did not appear thickened.

The histology of the adductor longus-pectineus exostosis formed in rats which had received BAPN for 1 week was found to be much like the description given by Yeager and Hamre (46). The most noticable change in the periosteum at the adductor

longus-pectineus insertion at 1 week was the increased cellularity of the inner layer. Immediately under the outer layer of periosteum the periosteal cells of the inner layer were seen to be hypertrophied with enlarged oval nuclei possessing prominent nucleoli. The cells were closely packed and relatively little connective tissue could be seen between them (Figure 24). This zone was called the proliferative zone by Yeager and Gubler (49).

Deep to the highly cellular region of the inner periosteum, the collagen fibers were more abundant and the periosteal cells were scattered among the connective tissue fibers. Large areas of amorphous intercellular material, perhaps preosseous tissue, could be seen in this area of the exostosis (Figure 25). This area was seen to gradually blend with the deepest portion of the periosteum composed of newly formed bone spicules and areas of new marrow tissue (Figure 26). The cells of this part of the enlarged periosteum were osteoblasts bordering on the bone spicules and premarrow cells seen in between the spicules. Usually a small blood vessel could be seen in the middle of each premarrow area.

The exostoses from animals fed the normal protein diet and given BAPN for 2, 3, or 4 weeks showed a continuing increase in size. The appearance of the fibrous portion of the exostoses had the same general histological structure as those from 1-week lathyric rats. The primary difference between 2-, 3-, or 4-week exostoses and 1-week growths was an increased amount of bone. The growth of the 2-, 3-, or

4-week exostoses was due to the large amount of intramembranous bone being formed, and there was relatively little or no increase in the size of the proliferative and prebone areas (Figure 27).

The exostoses from rats given a normal protein diet and BAPN for 5 or 6 weeks were very large. At this stage of development the growth at the insertion of the adductor longus and pectineus was composed almost entirely of bone possessing large marrow spaces. There was much osteoclastic activity in the marrow spaces showing that the marrow spaces within the exostosis were being enlarged and remodelled as the bony portion increased in size. In many of the 5-and 6-week exostoses the marrow spaces could be seen to be continuous with the central marrow cavity of the femur (Figure 28).

The fibrous part of the exostoses from 5-or 6-week normal protein-fed lathyric rats appeared much smaller than the comparable area in younger exostoses. A few hypertrophied periosteal cells were present; but large collagenous fibers were much more prominent than seen in the 2-, 3-, or 4-week exostoses. The connective tissue fibers could be seen extending from the bone within the exostosis out into the tendon of the adductor longus.

The osteogenic area of the 5- and 6-week exostoses from lathyric rats given the normal protein diet was considerably different from the prebone areas in younger exostoses. In the 2-, 3-, or 4-week exostoses the area of intramembraneous bone

formation was quite extensive but in 5- and 6-week exostoses there was very little intramembranous bone deposition. The bone appeared to be growing by appositional osteoblastic activity along the sides of Howship's lacunae and on the surface of the bone between Howship's lacunae.

In a few of the 5- and 6-week exostoses from Group 2 animals the prebone zone was seen to contain small patches of cartilage. In such areas the exostoses appeared to be undergoing endochondral ossification. The hypertrophied periosteal cells looked like mesenchymal cells and apparently possessed the capacity to differentiate into either osteoblasts or chondroblasts. Occasionally a mass of cartilage containing encapsulated chondrocytes could be seen at tips of newly formed bone spicules (Figure 29). Sections stained with azure A showed that each chondrocyte was surrounded by an intensely metachromatic matrix; but the spaces between chondrocytes had a mottled metachromatic and non-metachromatic appearance. The non-metachromatic areas appeared to be composed of immature bone (Figure 30). When cartilage was present in an exostosis it was usually confined to one small area and often appeared to be completely surrounded by immature bone (Figure 31).

In the areas of rapidly growing BAPN-induced exostoses where new bone was being formed, numerous small basophilic granules in the matrix of the bone could be seen in hematoxylin-eosin sections.(Figure 32). These small globules were also evident in sections stained with Azure A in which case they

stained metachromatically (Figure 33). They varied in size from about 1 micron to 5 micra. They were most prevalent at the ends of the growing bone trabeculae or in the center of the trabeculae. The globules were scarce or completely absent from the appositional bone which had been laid down by osteoblasts on the surface of the new trabeculae.

The Exostoses of Lathyric Rats Fed Low Protein Diet

When adult rats were started on a low protein diet and BAPN administration at the same time (Group 4) the exostoses were very much like the exostoses formed in normal protein-fed lathyric rats. Overall these exostoses were smaller than those in rats given BAPN and maintained on a normal protein diet. However, a marked variability in size of the exostoses was rather characteristic of this low protein-fed group.

For the first 2 weeks on the regimen the growth and the histology of the exostoses from Group 4 animals were similar to those of lathyric rats fed a normal protein diet. But at 3 weeks some differences were evident. The most noticable difference was the smaller size of the Group 4 exostoses. Histologically it was seen that there had been much less bone formed in the exostoses from Group 4 rats than was formed in those from Group 2 animals. The fibrous portion of the exostosis contained large areas of collagenous tissue, few hypertrophied periosteal cells and many smaller fibroblasts. The only osteogenic activity appeared to be appositional growth at ends of the bone spicules.

After 4 weeks on the low protein diet and BAPN administration the exostoses also were small and contained very little newly formed bone. However, a single 4-week exostosis from a Group 4 rat was much larger than the others. This particular growth was nearly as large as those seen in normal protein fed lathyric rats and was much larger than any 5-or 6-week exostosis from Group 4 animals. Histologically, it appeared as a typical rapidly growing adductor longus-pectineus exostosis (Figure 34).

When the animals of Group 4 had been on the experiment for 5 or 6 weeks they were seen to be only about half the size of the exostoses from lathyric rats fed the normal protein diet. The portion of the exostosis external to the bone in a typical 5- or 6-week Group 4 exostosis was seen to be composed of connective tissue with relatively few fibroblasts. It contained very little osteogenic tissue and very little newly formed bone (compare Figure 35 and Figure 36).

The animals of Group 6, which had been partially depleted of protein before BAPN was given, had the smallest adductor longus-pectineus exostoses of any of the rats studied in this experiment. The response of the periosteum to the BAPN appeared to be considerably lessened in animals given a low protein diet.

After the first week of BAPN the animals of Group 6 were seen to have slightly developed exostoses. The inner layer of periosteum at the adductor longus-pectineus insertion was

thickened. But it did not contain a distinct proliferative zone of hypertrophied periosteal cells. Some of the periosteal cells appeared enlarged but they were scattered among the connective tissue fibers and were not as a definite cellular zone. At 1 week no evidence of intramembranous bone formation was noted.

The fibrous portion of the exostoses from Group 6 rats given BAPN for 2, 3, or 4 weeks had the same general histological appearance as the 6-week exostoses from Group 4 animals. The outer portion of the exostosis was composed of large bundles of connective tissue, few typical periosteal cells with large oval nuclei, and numerous small fibrocytes. Some of the 2-, 3- and 4-week exostoses of Group 6 exhibited areas of intramembranous bone formation but never to the extent of those from Group 2 animals.

The 5- and 6-week exostoses from Group 6 rats were usually only slightly larger than the periosteum of the normal adductor longus-pectineus insertion sites. But they appeared quite different microscopically (compare Figure 37 to Figure 18). The cap of fibrous tissue overlying the bone of the exostosis was composed of large collagen bundles which appeared swollen. Closely associated with the large collagen bundles were seen cells that were smaller than the typical periosteal cells; they looked like the fibroblasts seen in the outer layer of periosteum and in the adductor longus tendon (Figure 38). The large bundles of collagen appeared to be attached to the

bone of the exostosis usually parallel with the direction of muscle pull and therefore appeared to be related to the tendon. The spaces of the exostosis not occupied by large collagen bundles contained smaller connective tissue fibers and typical periosteal cells with large oval nuclei (Figure 39).

Very little bone had formed in the 5- and 6-week exostoses from Group 6 animals. Any bone which had formed was accomplished by appositional growth on the surface; thus the bone was not trabecular and contained no large marrow spaces (Figure 40 and Figure 41).

The Comparison of Exostosis Size of Normal Protein-Fed and Low Protein-Fed Lathyric Rats

In order to compare the sizes of the pectineus-adductor longus exostoses produced by BAPN, the largest cross-sectional areas of each were used as an index of relative size. This was done by projecting the image of the cross-section onto a paper of uniform thickness with a microprojector. The image of the exostosis was traced and each tracing was cut out and weighed. The weights obtained in this manner appear in Table 5. The outlines of the exostoses in cross-section may be seen in Figure 2.

Sections of 4 different exostoses were taken each week from each of the BAPN treated groups of rats. Group 2 animals were given BAPN and fed a normal protein diet; Group 4 rats were started on a low protein diet and BAPN at the same time; and Group 6 consisted of rats given a low protein diet 4

weeks before starting on BAPN, then maintained on low protein and BAPN for the duration of the experiment.

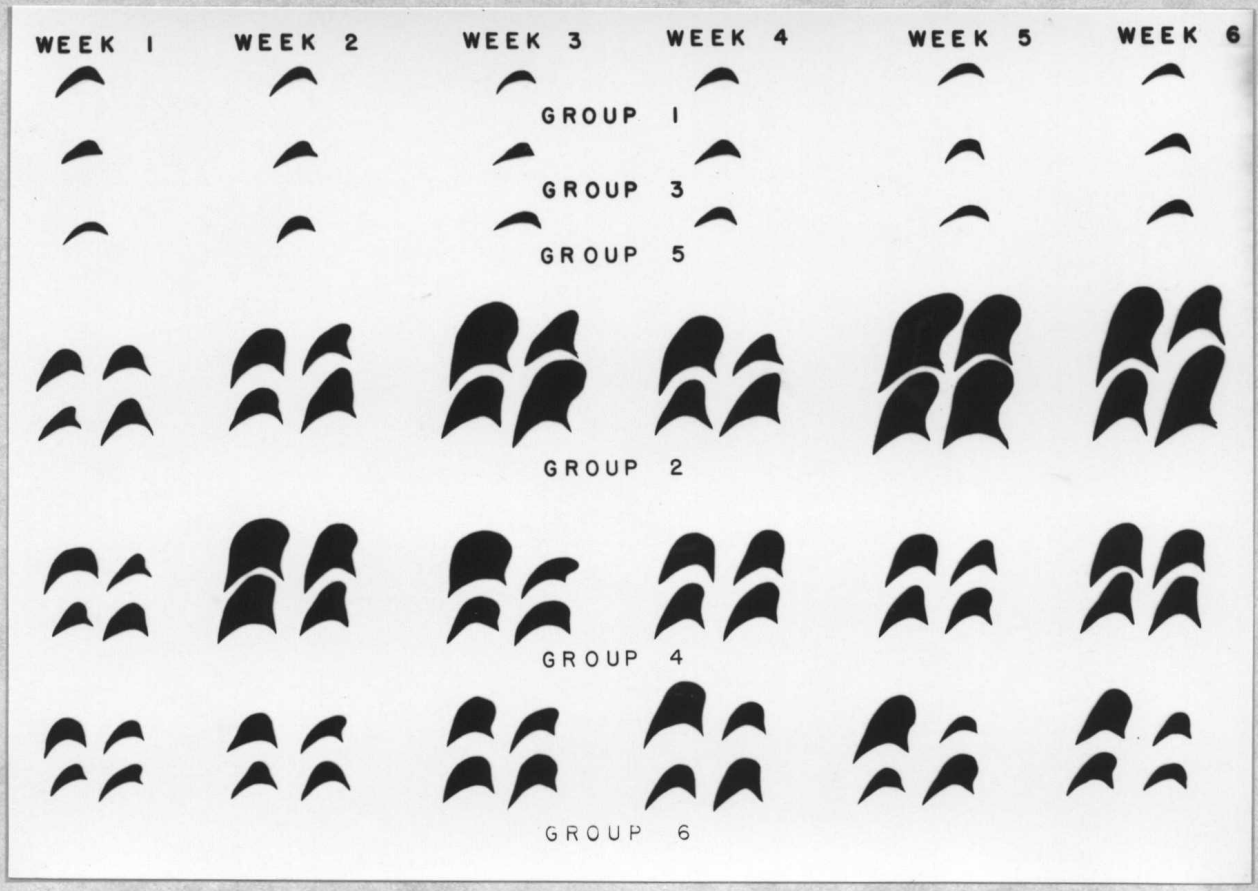

FIGURE 2. - Cross-sectional areas of the adductor longus-pectineus insertion sites of the rat femurs. Group 1, normal protein diet; Group 2, normal protein diet plus BAPN; Group 3, low protein diet; Group 4, low protein diet plus BAPN; Group 5, low protein diet for same length of time as Group 6; Group 6, low protein diet for 4 weeks, then low protein diet plus BAPN. The weeks indicate the length of time on experiment.

Only a single cross-section of the pectineus-adductor longus insertion was taken for each week from the control rats since they were so uniform in size. Group 1 rats were fed a normal protein diet. Group 3 animals were fed a low protein diet and Group 5 animals were maintained on a low protein diet for the same length of time as the Group 6 rats.

Compare the cross-sectional areas of the various groups and expecially note the difference in size between those of Group 2 and Group 6.

TABLE 5. - The weights of the paper cutouts which are tracings of the outline of the adductor longus-pectineus insertions.

Week	Group	Weights of cross sections in milligrams				Average Weight
1	1	49.4				49.4
	2	92.4	97.4	56.0	83.4	82.3
	3	41.8				41.8
	4	104.0	122.4	65.0	56.6	87.0
	5	27.4				27.4
	6	84.2	50.0	61.4	52.0	61.9
2	1	41.6				41.6
	2	190.0	111.6	150.0	185.6	125.6
	3	42.4				42.4
	4	128.6	318.0	137.0	99.4	170.8
	5	40.8				40.8
	6	94.0	76.0	70.6	111.4	88.0
3	1	27.4				27.4
	2	241.2	165.6	402.8	377.0	296.7
	3	35.0				35.0
	4	176.6	167.4	148.4	166.0	164.6
	5	38.2				38.2
	6	146.0	159.6	146.0	94.6	136.6
4	1	40.1				40.1
	2	159.6	201.4	94.4	309.0	191.1
	3	42.4				42.4
	4	321.8	332.0	149.2	260.0	240.8
	5	36.8				36.8
	6	92.0	158.6	110.0	234.4	149.0
5	1	34.0				34.0
	2	345.4	487.0	450.0	331.6	403.5
	3	36.0				36.0
	4	119.6	162.6	125.0	94.0	125.3
	5	34.0				34.0
	6	250.6	145.0	71.6	44.4	128.0
6	1	25.4				25.4
	2	249.0	409.4	416.0	205.6	320.0
	3	38.4				38.4
	4	230.0	169.2	167.6	158.4	181.3
	5	44.0				44.0
	6	68.0	114.6	64.0	196.0	110.7

CHAPTER IV

DISCUSSION

In the study of any disease process produced in an experimental animal numerous factors, some directly related to the causative agent and others seemingly unrelated to the agent, affect the rate and course of the disease. Osteolathyrism in the rat is no exception. The course of experimental lathyrism in the rat whether caused by feeding Lathyrus peas or administering BAPN is invariably modified by several factors; the age of the animal, the length of time of administration of the lathyrogenic agent, the sex of the animal and the amount and type of dietary protein.

The age of the rat when exposed to the lathyrogenic factor is one of the most important variables in this disease. When given to weanling rats for an extended time BAPN will cause collapse of vertebrae, disorganization of epiphyseal plates, enlargement of costochondral junctions, exostoses, herniations and angiorrhexis. Yet, adult rats given BAPN will develop exostoses and epiphyseal plate lesions as rapidly as the young animals; but they will not develop the other symptoms (60).

The length of time that rats are fed the sweet pea meal or given BAPN markedly affects the severity of the symptoms

of lathyrism. The longer the lathyrogenic agent is given, the greater the skeletal deformities become whether the rat is young or is an adult. In weanling rats dissecting aneurysms are often found to occur after 4 weeks of lathyrus treatment but some of the skeletal changes occur within 1 or 2 days after initiation of treatment. When the lathyrogenic stimulus is stopped the skeletal lesions cease to progress.

The amount of protein and the nature of the protein in the diets of lathyric rats has been demonstrated to be an important factor that may modify the course of the disease. The incidence of aortic rupture in weanling rats was reported to be much higher when they received a 12 per cent casein diet than when they were fed an 18 per cent casein diet (27). Yet, more weanling rats given Purina rat pellets (23 per cent protein) developed aortic aneurysms than did those fed an 18 per cent casein diet (58). It appears, therefore, that the type of protein fed may determine the nature of the response to BAPN. In the present investigation the diets used contained casein as the protein. It was deemed advisable to use just one type of protein for both the adequate protein diet and the low protein diet. In this way the factor of different types of protein could be eliminated.

In this study lowering the amount of dietary protein to 8 per cent did not alter the histology of the aortas of adult lathyric rats. There may be several explanations for this. Perhaps the aortas from adult lathyric animals are not as

sensitive to dietary protein levels as are the aortas from weanling rats, or the adult aorta is changed enough from that of the weanling rat to be no longer responsive to the lathyrogenic agent, no matter what extraneous factors are introduced.

If it is true that the adult rat aortas are no longer affected by BAPN, there must be some basic difference in these vessels in the adult and the young rats. Walker (36) has suggested that the lathyrogenic agent acts in young rats by inhibiting the formation of new elastic fibers and does not affect pre-existing ones. If this is the case, it would be expected that the aorta of a young rat which is rapidly growing and remodelling would be vulnerable to anything that inhibits elastic fiber formation. In contrast, the aorta of a 60-day old rat would not be growing or remodelling to any great extent; and thus it would not be affected by a lathyrogenic agent. Gillman (41) supported this view by stating that the rat aorta is most sensitive to the lathyrogenic toxin when the rat is between 25 and 30 days of age, the age when the aorta is growing at its greatest rate; and thus the greatest amount of remodelling of elastic membranes is taking place.

If the reason for the failure of dissecting aneurysms to develop in the aortas of the adult rats fed the low protein diet was due to a decrease in sensitivity of adult aortas to low levels of dietary protein, it is possible that dissecting aneurysms would have been produced in the present experiment

if the animals had been maintained on the low protein diet and BAPN for longer periods of time. Decreasing the percentage of protein in the diet to an even lower level may also result in aortic changes if the difference between the adult and weanling rat aortas is a matter of degree of sensitivity.

In this study the pectineus-adductor longus exostoses which were formed in Group 2 BAPN treated rats fed a normal protein diet were produced by the processes of proliferation of periosteal cells and osteogenesis and continued to grow for the duration of the experiment. However, the exostoses in the animals which had been on the experiment for 5 or 6 weeks gave the appearance of having ceased to grow. These exostoses were composed almost entirely of bone and a very small proliferative zone. The portion of the exostoses above the bone was composed of dense connective tissue with relatively few typical proliferating periosteal cells. Proliferating periosteal cells were seen to comprise the entire outer portion of the inner periosteum of the younger exostoses of animals which received BAPN for 1 to 3 weeks.

Patches of cartilage were found in a few of the larger exostoses of Group 2 rats. The formation of cartilage in an exostosis always appeared to be related to the size of the growth. Cartilage was found only in exostoses which were growing more rapidly than other exostoses. A possible explanation of the presence of cartilage may be related to the rapid growth. The formation of cartilage has been stated

to occur in areas where the blood supply is not very extensive (61); if the exostoses are growing so fast that adequate blood supply cannot keep pace, the periosteal cells may differentiate into chondroblasts instead of osteoblasts. This appears to have occurred in some specimens of this study.

The areas of cartilage in exostoses of this study were always located at the tips of the newly formed bone spicules and were never found in the center of the growth. The location of the cartilage may be explained by the unique blood supply of the periosteum at the site of the adductor longus insertion. A large share of the blood supply to the periosteum in this area comes from vessels which travel the length of the tendon from the muscle to end in a network in the periosteum (62). Thus it would be expected that the center of the exostosis where the adductor longus inserts would receive a rather extensive blood supply. However, the areas in which cartilage was found in the specimens of this study did not possess large blood vessels and appeared relatively avascular.

Why the exostoses formed in BAPN-treated rats maintained on an 8 per cent casein diet were much smaller than those from lathyric rats fed a 27 per cent casein diet is difficult to explain. It was thought that perhaps they were smaller only because the low protein-fed rats were smaller and were not actually relatively smaller. In order to determine if this was the case, the weights of the exostosis cross-sectional cutouts per body weight from Group 2 rats were compared with the weights of the exostosis cross-section cutouts per body

weight from Group 4 and Group 6 rats. Using the weight of the cross-section paper cutout as a reflection of the total size of the exostosis, it was found that the ratio of exostosis size to total body weight for the low protein-fed lathyric rats was much lower than for the normal protein-fed lathyric rats. This indicated that the exostoses from Groups 4 and 6, animals fed the 8 per cent protein diet, were actually and proportionately much smaller than those from Group 2 rats fed the 27 per cent protein diet.

Observations made during the course of the experiment indicated that the amount of food eaten per day was approximately the same for all of the rats regardless of whether they ate the low protein diet or the normal protein diet. So it was assumed that the dietary intake by itself was not a factor in the differences in size of the exostoses of Group 2 animals and those of Group 4 and 6.

The animals of the experiment maintained on the low protein diet exhibited no signs of amino acid deficiency such as fatty or cirrhotic livers, cataracts, etc. They appeared healthy and active, and were gaining some weight. Therefore, it would appear that results of differences in the periosteal response to BAPN between low protein-fed animals and those receiving normal dietary protein could not be explained on a purely protein deficiency basis.

Yeager, et al. (63) reported that rats restricted to a limited food intake either had small exostoses or no exostoses. This finding may be related to results of the present investi-

gation since a restriction of total food intake would lower the amount of dietary protein.

It may be seen from Figure 2 that the exostoses from Group 4 rats given the low protein diet and BAPN were as large as those from Group 2, animals fed the 27 per cent protein diet, for the first 2 weeks. The Group 6 animals which were placed on the low protein diet before being given BAPN never had exostoses as large as those from Group 2 or Group 4. From this finding it would appear that some factor related to the level of dietary protein intake was active in modifying the response of the periosteum to the lathyrogenic agent. The animals of Group 4 were started on the low protein and the BAPN at the same time. Thus, these animals had received an adequate protein diet right up until they first were given the lathyrogenic agent; and apparently some residual effect of the adequate protein diet was effective in producing a nearly typical response of the periosteum to the BAPN. But after 3 weeks on the low protein this effect was no longer present, as was evident from the cessation of proliferation and bone formation.

Group 6 rats were started on the 8 per cent casein diet 4 weeks before BAPN was given; and this group of animals had very small exostoses that never showed the typical periosteal proliferative response characteristic of lathyrism in adult rats. Apparently placing the animals on a low protein diet before administering BAPN removed some factor

required for the usual exostosis formation in lathyrism. Perhaps even a longer period on low protein before giving BAPN would have resulted in complete inhibition of the formation of exostoses.

Low protein diets fed to weanling rats have been reported as causing an enhancement of the aortic and skeletal changes of lathyrism. Yet, the results of this investigation indicated that a low protein diet in some way caused a decrease in the exostosis formation in adult lathyric rats. Although these findings were contradictory, they both suggested that protein metabolism is closely associated with the effects of lathyrogenic agents.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

This investigation was designed to test the effects of a low protein diet on the production of aortic aneurysms and exostoses by BAPN. Seventy-eight adult female albino rats 60 days of age and an average body weight of 172 grams were employed in this investigation. Control animals were fed an adequate protein diet of 27 per cent casein while experimental animals were fed an 8 per cent casein diet. The BAPN was supplied in drinking water at the rate of 150 milligrams per 100 millileters water.

The animals were divided into 6 groups. Group 1 composed of 12 rats was fed the 27 per cent casein diet, the 12 rats of Group 2 were fed the 27 per cent casein diet and given BAPN; the 12 rats of Group 3 were fed the 8 per cent casein diet; Group 4 composed of 12 rats was fed the 8 per cent casein diet and given BAPN; the 12 animals of Group 5 were fed the 8 per cent casein diet; Group 6 composed of 18 animals was fed the 8 per cent diet for 4 weeks and then given BAPN and continued on the low protein diet.

Two rats from each of Groups 1, 2, 3 and 4 were killed weekly beginning at the end of the first week until all animals

were killed. The animals of Group 5 were killed at the rate of 2 rats each week beginning at the end of the fifth week of low protein feeding. Two animals from Group 6 were killed each week at the end of the fifth, sixth and seventh week of low protein feeding in which case they had been given BAPN for 1, 2 and 3 weeks. Four rats were killed each week from that point until the end of the experiment.

The animals from all of the groups appeared active and healthy throughout the experiment. The adult rats given the low protein were found to gain weight much more slowly than those which received an adequate protein diet. However, the administration of BAPN to either those fed a low protein diet or a normal protein diet did not appear to effect appreciably the weight gain.

All animals were killed with ether and subjected to autopsy examination. The entire thoracic aorta was removed and placed in fixative. Both femurs with the attached pectineus and adductor longus muscles were removed, fixed and decalcified. The thoracic aorta and femurs were prepared for microscopic examination. The histological sections of the aorta were stained with Harris' hematoxylin-eosin, Masson's trichrome, Gormori's aldehyde-fuchsin, periodic acid-Schiff, alcian blue, alcian blue-PAS and hematoxylin-eosin, periodic acid-Schiff and Azure A.

The histological appearance of the thoracic aorta of the adult rat was presented. The aorta in the rat was noted to

possess a very thin tunica intima, a prominent tunica media and relatively thin tunica adventitia sharply separated from the periaortic fat. The histology of the thoracic aorta was seen to be very uniform except at sites where arterial branches were given off and where the ligamentum arteriosum attached. No histological differences could be seen in the aortas from the different groups of animals. The maintainance of adult lathyric rats on a low protein diet for 8 weeks did not produce dissecting aneurysms of the aorta; nor were any histologically demonstrable alterations suggestive of weakening of the vessel evident.

Exostoses at the site of insertion of the adductor longus and pectineus on the femur were noted in all rats given BAPN. However, a marked difference in the size of the exostoses produced by BAPN was noted in the various groups of experimental rats.

Microscopic sections of the exostoses from rats maintained on a normal protein diet and BAPN (Group 2) for 1 week appeared much like the descriptions of Yeager and Hamre (46). The inner layer of periosteum at the insertion was enlarged due to a proliferation and hypertrophy of the periosteal cells, an increase in connective tissue fibers and an intramembranous formation of bone.

The exostoses from rats given normal protein and BAPN for 2, 3 or 4 weeks showed a progressive enlargement and no remarkable histological changes except an increased amount

of newly formed bone with well developed marrow spaces.

After 5 or 6 weeks on the normal protein diet and BAPN, the animals had very large adductor longus-pectineus exostoses. They were composed mostly of bone with large marrow spaces; and external to the bone, a fibrous layer containing hypertrophied periosteal cells was seen. A few of the large exostoses contained small patches of cartilage.

The exostoses in rats started on the low protein diet and BAPN simultaneously (Group 4) appeared much like those from animals fed an adequate protein diet for the first 2 weeks on the experiment. But after 3 weeks, development of the exostoses appeared arrested. Arrest was due to reduction in number and disappearance of proliferating hypertrophied periosteal cells and cessation of osteogenesis except by slight appositional growth at the surface of cortical bone at the muscle insertion.

The animals of Group 6 which had been fed the low protein diet for 4 weeks before they were started on BAPN were found to have very small exostoses throughout the experiment. After 1 week on BAPN the exostoses were not much larger than the periosteum of the normal insertion sites and very little proliferation and hypertrophy of the periosteal cells was evident.

After 2, 3 and 4 weeks the exostoses from Group 6 rats were very small. They did not possess a distinct area of proliferating periosteal cells and showed little or no intramembranous bone being formed.

At 5 and 6 weeks the exostoses from Group 6 animals were also very small. They were seen to have many large collagen bundles and smaller periosteal cells that looked like typical fibroblasts. Very few periosteal cells with large oval nuclei were seen. No intramembranous bone formation was evident; but some bone was formed by appositional growth on the walls of Howship's lacunae and on the surface of the old bone.

In order to determine the relative sizes of the exostoses from the various groups the largest cross-sectional area of each exostosis was projected on to paper, traced, cut out and weighed. A comparison of the weights of the paper tracings of the exostosis cross-sections demonstrated the smaller size of the exostoses formed in low protein-fed lathyric rats when compared to the exostoses from lathyric rats given an adequate protein diet.

Conclusions

1. Adult female rats fed an 8 per cent protein diet gained weight at a slower rate than adult female rats fed a 27 per cent diet but at the end of a 6-week period of feeding remained active and healthy.
2. Administration of BAPN to rats fed the 8 per cent or the 27 per cent protein diet had little or no effect on gain in weight of either group of animals.
3. Dissecting aneurysms of the aorta did not appear in either the rats fed the 27 per cent or the 8 per cent protein diet, even after 8 weeks of feeding of the latter diet.
4. Administration of BAPN to rats fed the 27 per cent protein diet caused exostoses to appear at the common insertion to the femur of the adductor longus and pectineus muscles.
5. Adult rats fed the 8 per cent protein diet and administered BAPN (Group 4) developed exostoses which showed arrested growth after 2 to 3 weeks. Arrest was characterized by cessation of periosteal cells proliferation and a marked decrease in osteogenesis.
6. Feeding the low protein diet for 4 weeks to adult rats before starting BAPN administration (Group 6) resulted in a marked suppression or inhibition of exostosis formation and minimal bone growth.

7. Adductor longus-pectineus exostosis formation in adult lathyric rats fed the 8 per cent protein diet was arrested in all cases and completely inhibited in some cases.

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PLATE I

Figure 3. Photomicrograph of the endothelium of the aorta from a Group 6 rat. e, endothelial cell; i, internal elastic membrane. Masson's trichrome. X2100.

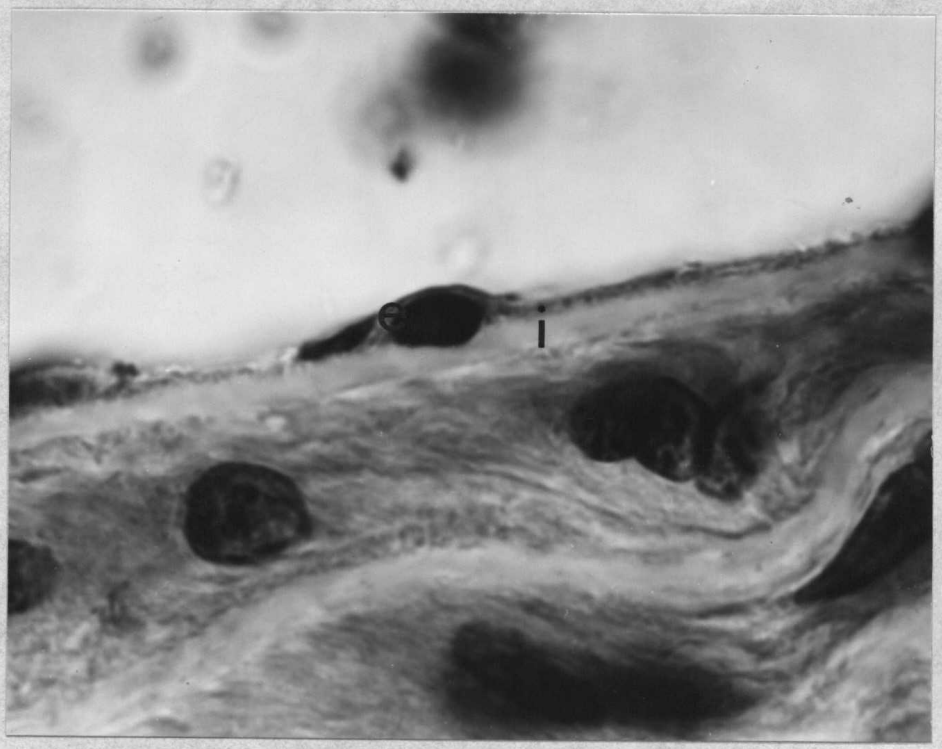

FIGURE 3

PLATE I

PLATE II

Figure 4. Photomicrograph of the endothelium of the aorta from a Group 6 rat. Note the smooth muscle cell immediately beneath the endothelial cell. e, endothelial cell; s, smooth muscle cell; i, elastic membrane. Hematoxylin-eosin-azure II. X2100.

Figure 5. Photomicrograph of the aorta from a Group 5 rat. Note the thickness of the elastic membranes toward the luminal side. l, lumen; p, periaortic fat; a, adventitia; m, media. Periodic acid-Schiff-hematoxylin. X205.

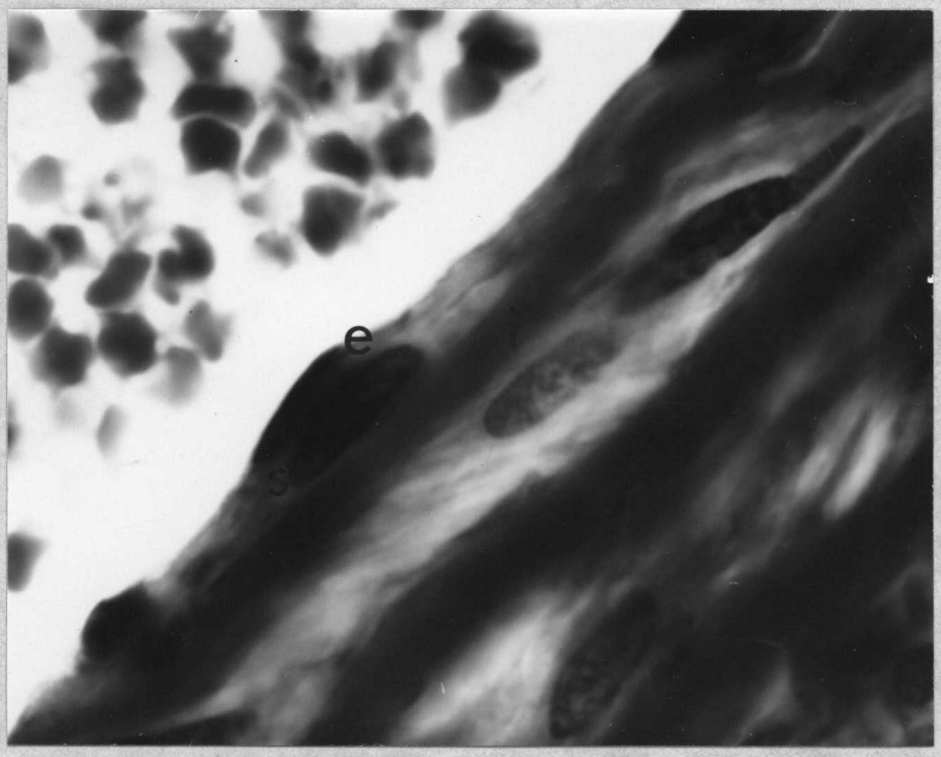

FIGURE 4

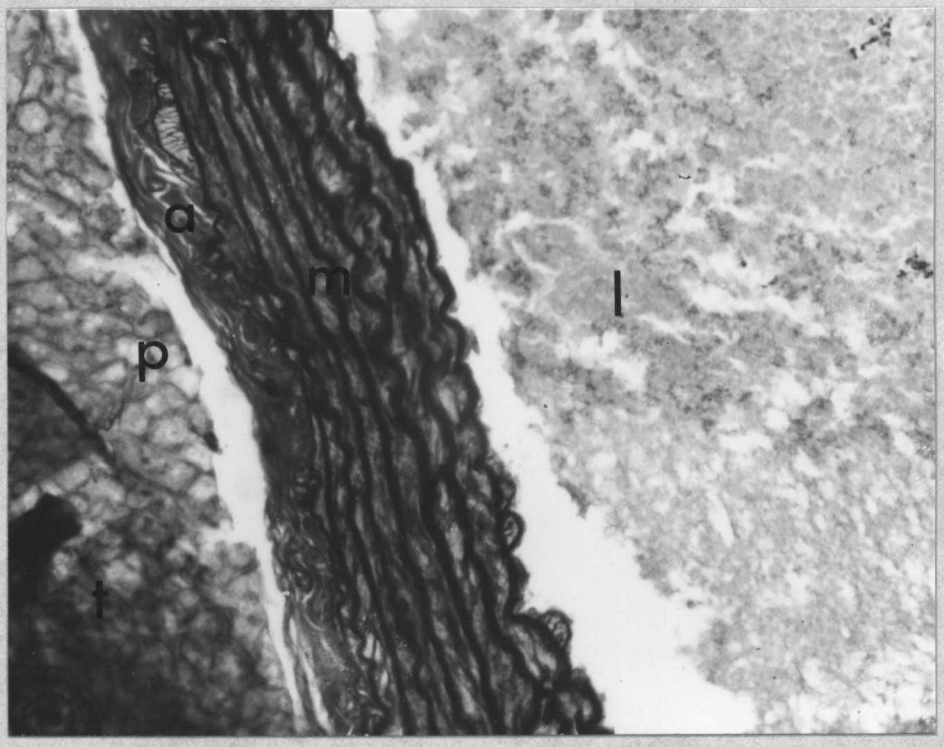

FIGURE 5

PLATE II

PLATE III

Figure 6. Photomicrograph of a cross-section of an aorta from a Group 6 rat. s, smooth muscle cell; i, elastic membrane. Hematoxylin-eosin-azure II. X900.

Figure 7. Photomicrograph of the media of the aorta from a Group 3 rat. Note elastic fibers branching from the elastic membranes. i, elastic membrane; f, elastic fiber. Gomori's aldehyde-fuchsin. X2100.

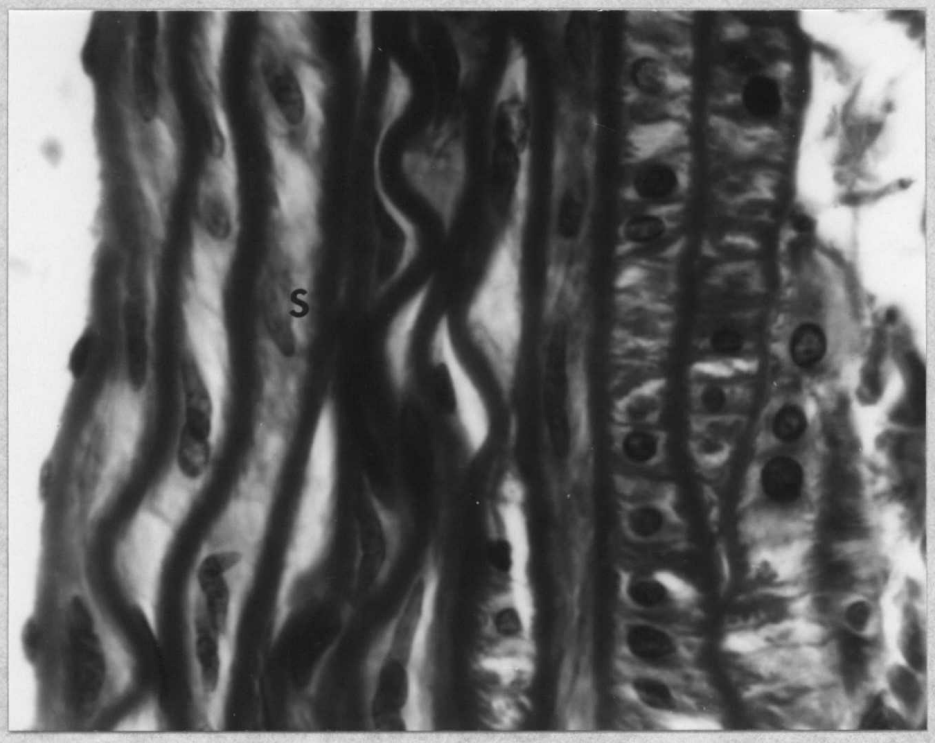

FIGURE 6

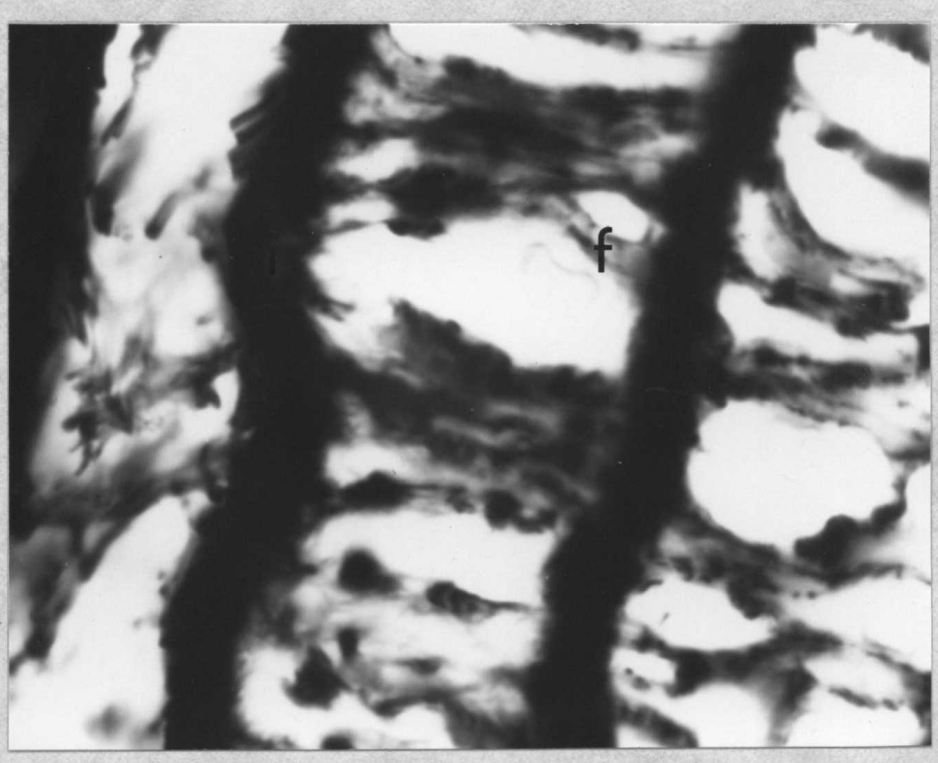

FIGURE 7

PLATE III

PLATE IV

Figure 8. Photomicrograph of the media of the aorta from a Group 4 rat. Note the reticular fibers between the smooth muscle cells. r, reticular fibers; s, smooth muscle cell. Periodic acid-Schiff-hematoxylin. X2100.

Figure 9. Photomicrograph of the media of the aorta from a Group 6 rat. Note the alcian blue positive material on the surface of the elastic membranes. i, elastic membrane. Alcian blue. X2100.

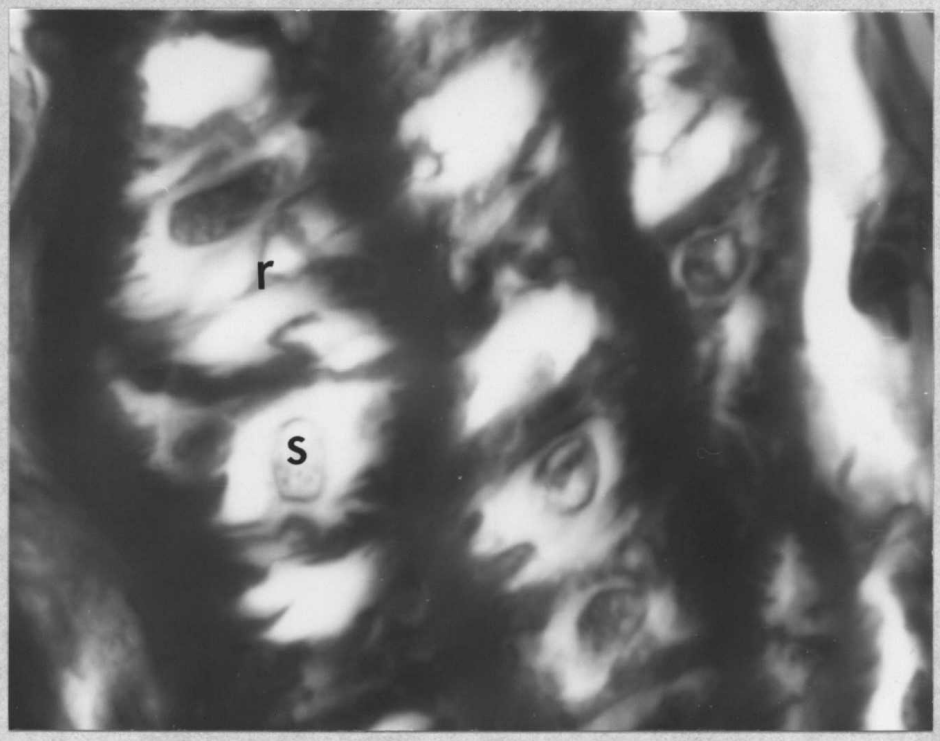

FIGURE 8

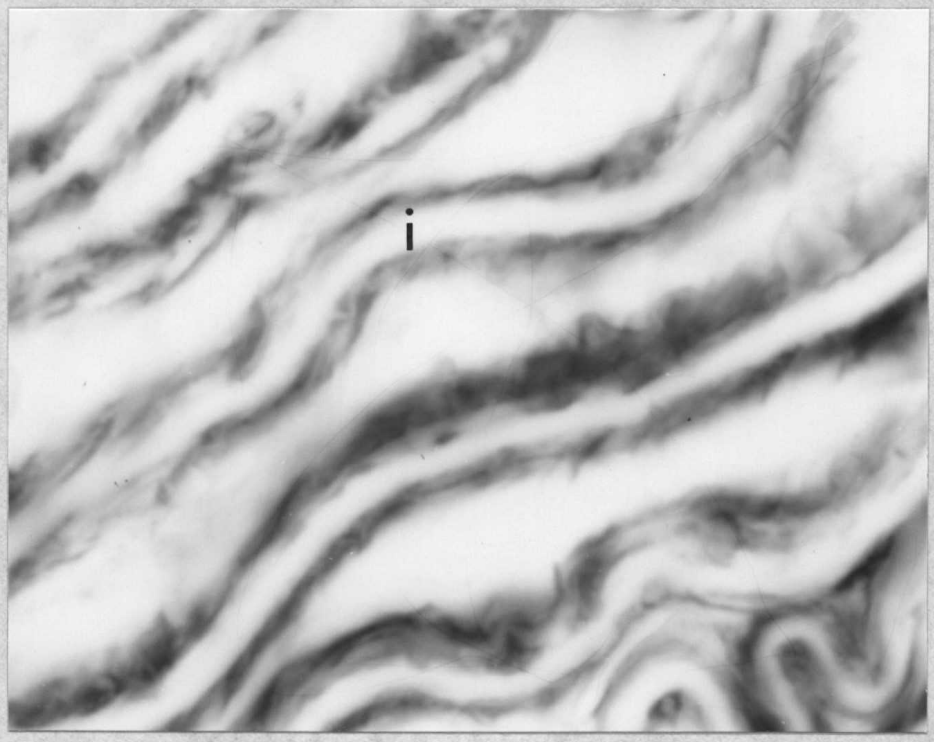

FIGURE 9

PLATE IV

PLATE V

Figure 10. Photomicrograph of a cross-section through the aorta from a Group 6 rat. Note separation of the adventitia from the surrounding fat. m, media; a, adventitia; p, periaortic fat; l, lumen. Masson's trichrome. X205.

Figure 11. Photomicrograph of the adventitia of the aorta from a Group 6 rat. x, external elastic membrane; f, fibroblast; v, vas vasorum; p, periaortic fat. Hematoxylin-eosin-azure II. X900.

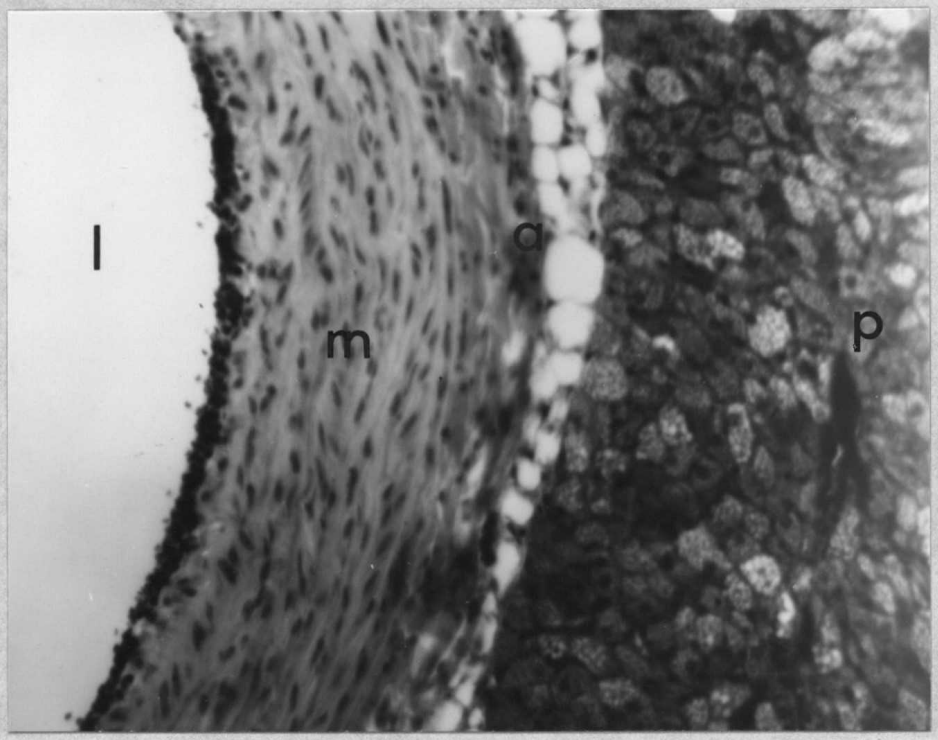

FIGURE 10

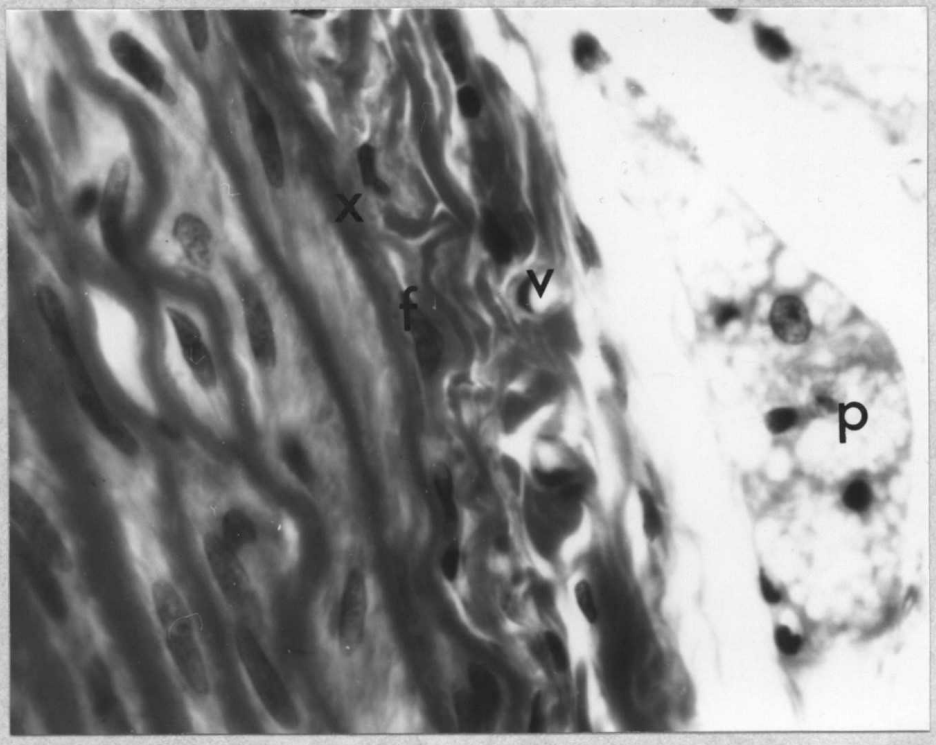

FIGURE 11

PLATE V

PLATE VI

Figure 12. Photomicrograph of the aorta from a Group 6 rat. Note the arrangement of the elastic membranes in the media at the point where the arterial branch is given off. ab, arterial branch; w, aortic wall. Hematoxylin-eosin-azure II. X205.

Figure 13. Photomicrograph of a cross-section of the aorta from a Group 6 rat. Note the ligamentum arteriosum attached to the aorta. o, ligamentum arteriosum; a, aorta; pa, pulmonary artery; n, recurrent laryngeal nerve. Gormor's aldehyde-fuchsin. X70.

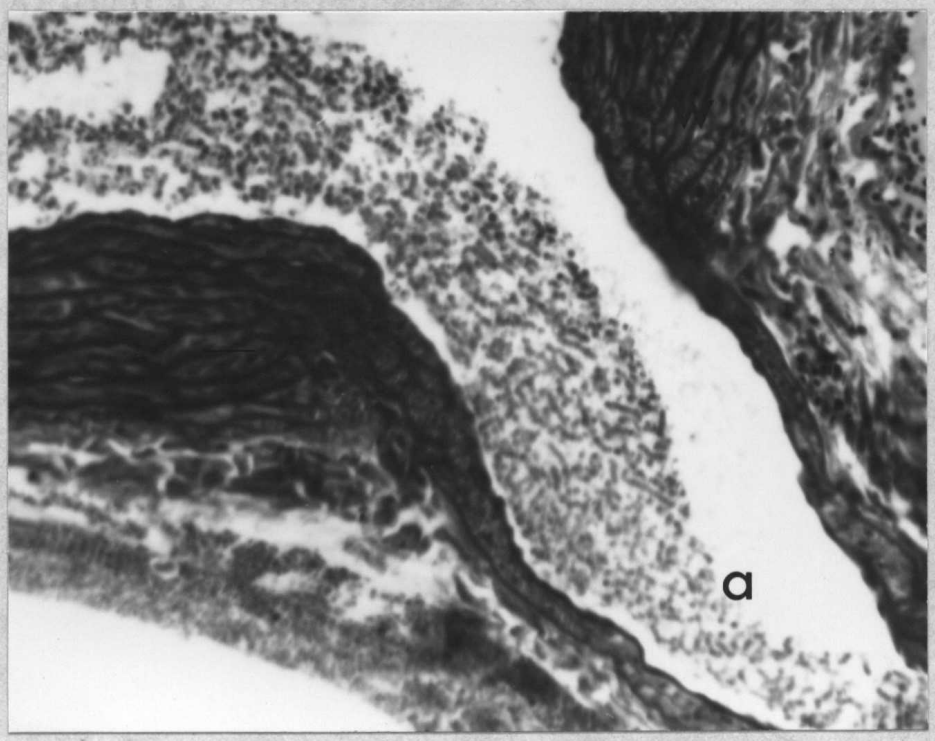

FIGURE 12

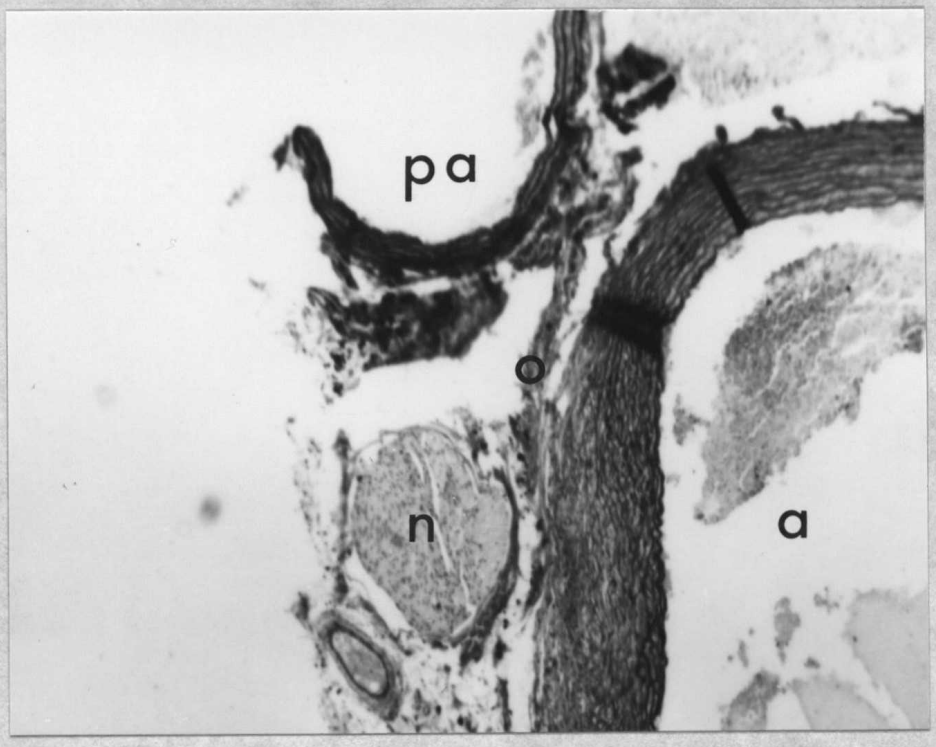

FIGURE 13

PLATE VI

PLATE VII

Figure 14. Photomicrograph of the aorta at the site of the ligamentum arteriosum attachment from a Group 6 rat. i, elastic membranes; c, collagenous fibers. Gomori's aldehyde-fuchsin. X205.

Figure 15. Photomicrograph of the aorta at the site of the ligamentum arteriosum attachment from a Group 6 rat. Note the fibroblasts in the media of the aorta. f, fibroblast; s, smooth muscle cell; c, collagen; i, elastic membrane. Masson's trichrome. X900

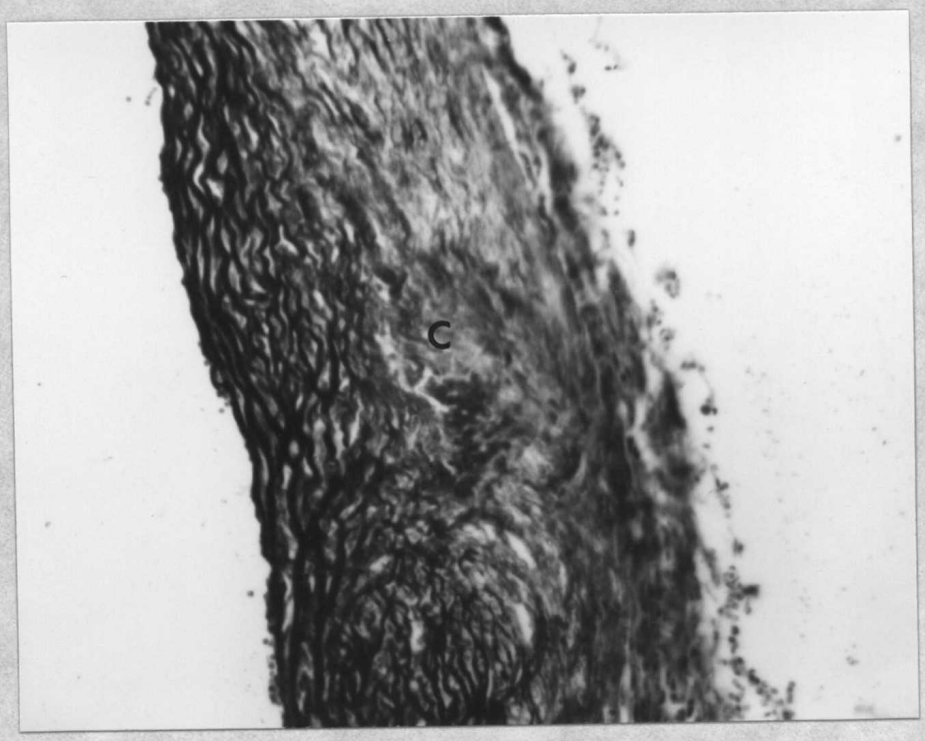

FIGURE 14

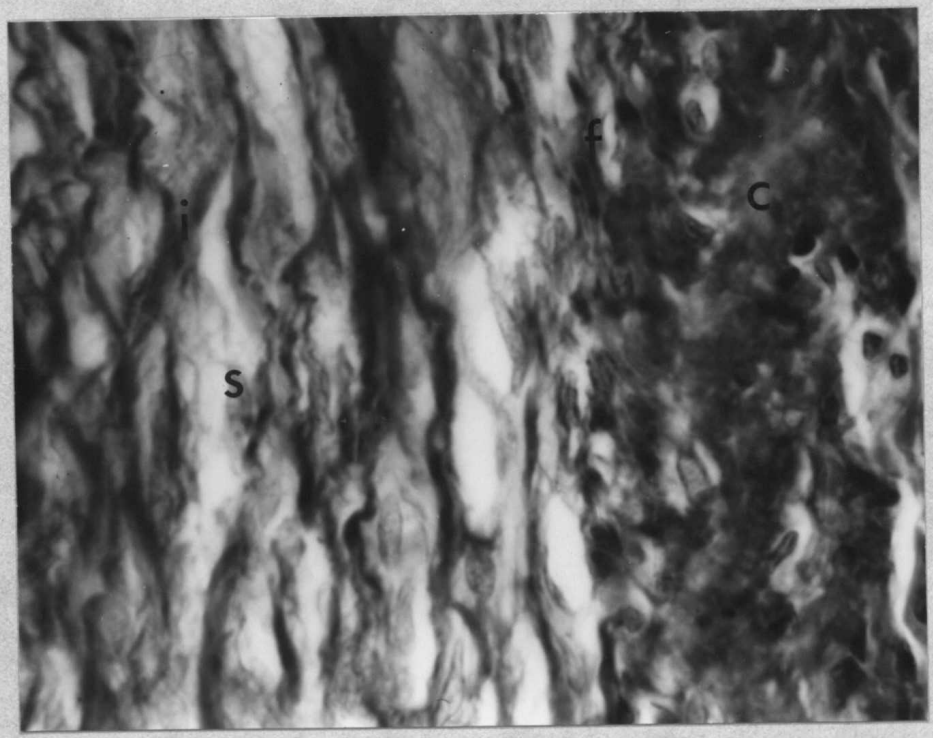

FIGURE 15

PLATE VII

PLATE VIII

Figure 16. Gross photograph of the femur with attached pectineus and adductor longus muscles from a Group 5 rat. f, femur; p, pectineus; al, adductor longus.

Figure 17. Photomicrograph of the normal periosteum of a femur from a Group 1 rat. b, bone; k, lacuna; p, periosteum. Hematoxylin and eosin. X2100.

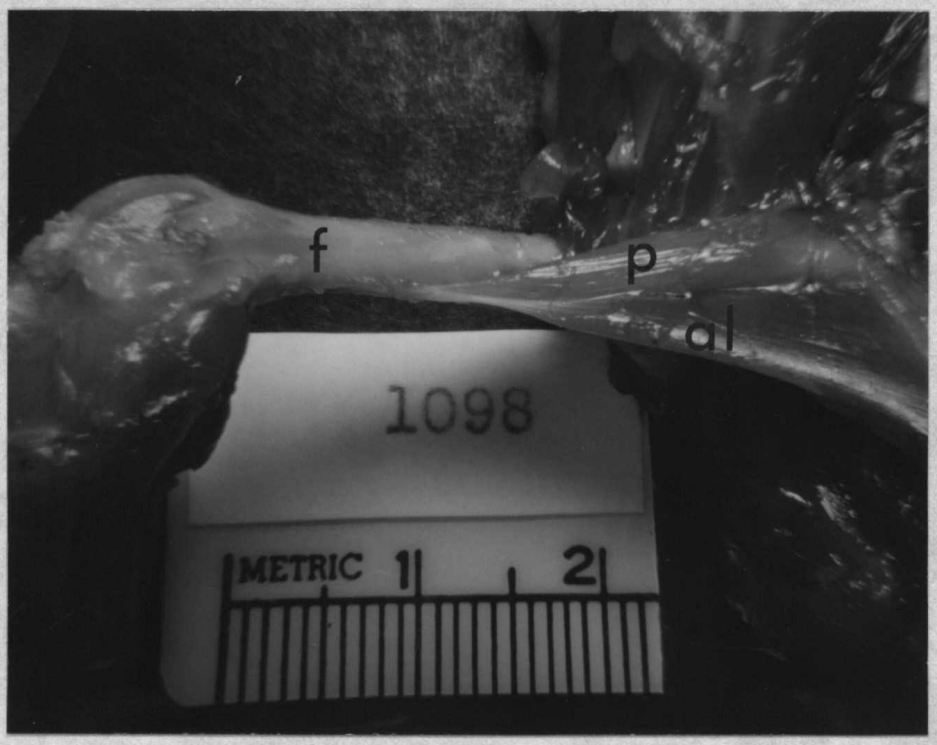

FIGURE 16

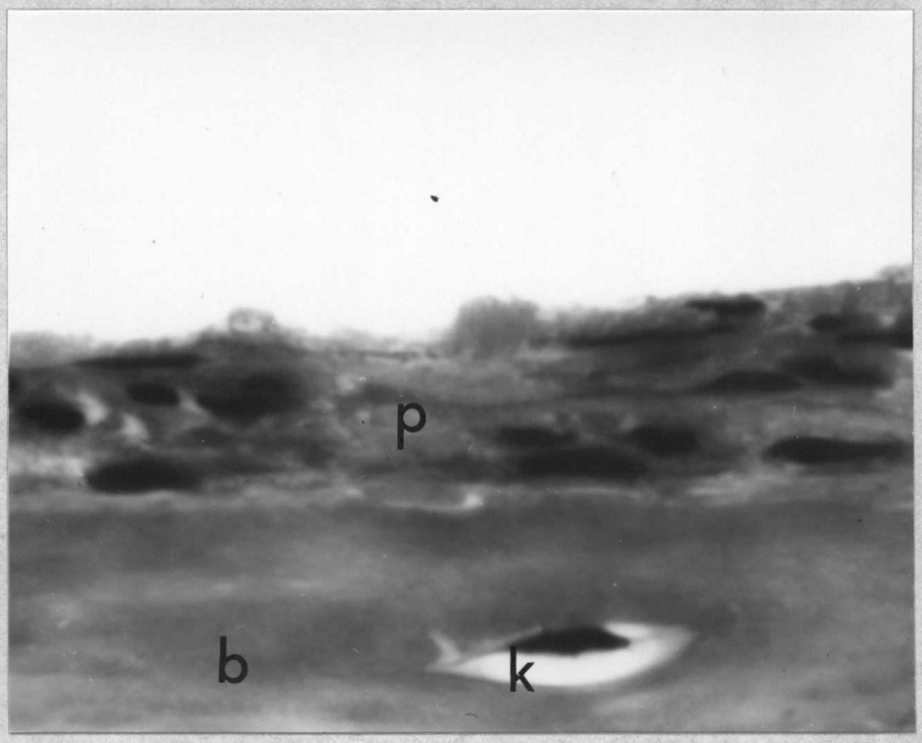

FIGURE 17

PLATE VIII

PLATE IX

Figure 18. Photomicrograph of a cross-section through a normal pectineus-adductor longus insertion site on a femur from a Group 1 rat. c, cortical bone; p, periosteum; al, adductor longus tendon; pn, pectineus. Masson's trichrome. X47.

Figure 19. Photomicrograph of the pectineus-adductor longus insertion from a Group 1 rat. Note the difference between the outer and inner periosteum. f, fibroblast; p, periosteal cell; op, outer periosteum; ip, inner periosteum. Masson's trichrome. X900.

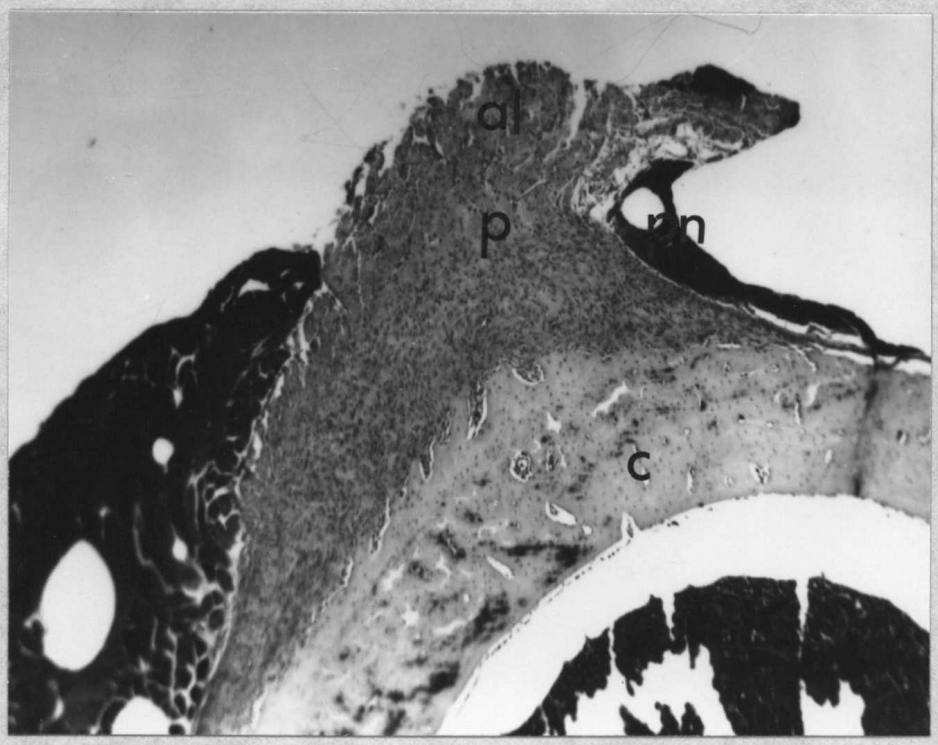

FIGURE 18

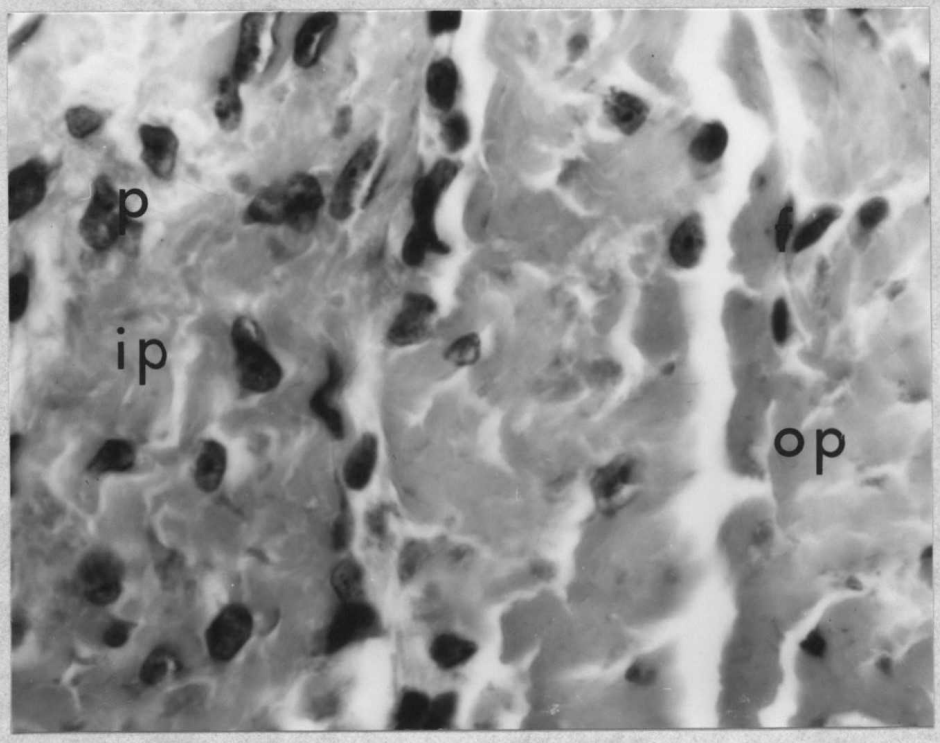

FIGURE 19

PLATE IX

PLATE X

Figure 20. Photomicrograph of the pectineus-adductor longus insertion from a Group 1 rat. Note how the outer periosteum blends with the adductor longus tendon. al, adductor longus tendon; pn, pectineus. Periodic acid-Schiff-hematoxylin. X47.

Figure 21. Photomicrograph of the cellular detail of the inner layer of periosteum at the pectineus-adductor longus insertion from a Group 1 rat. Note the large oval nuclei. Masson's trichrome. X900.

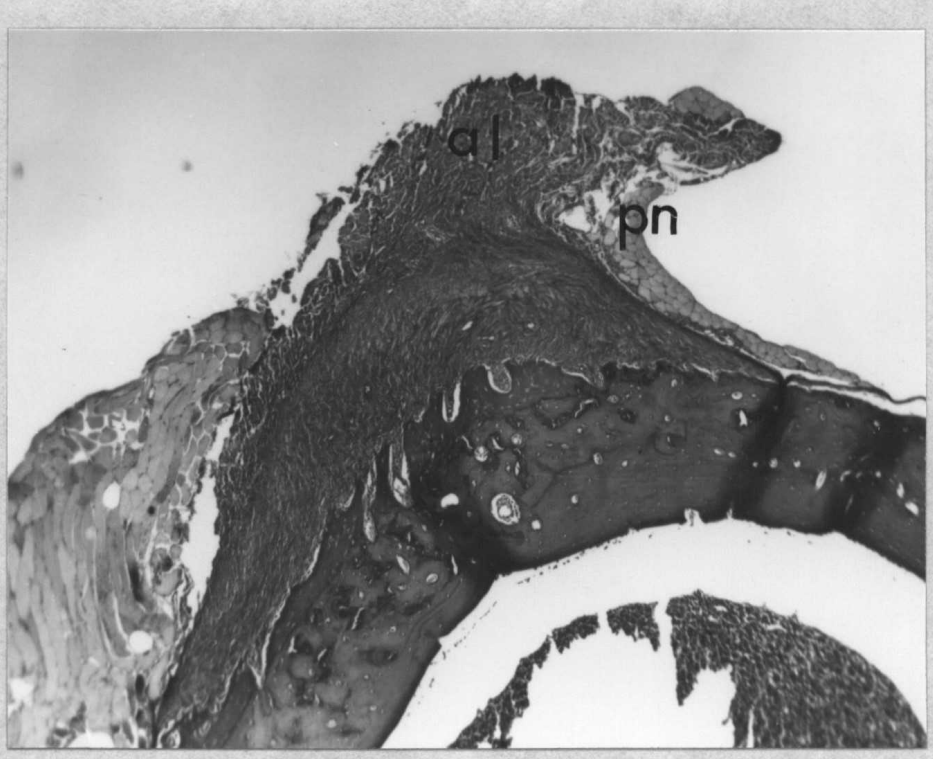

FIGURE 20

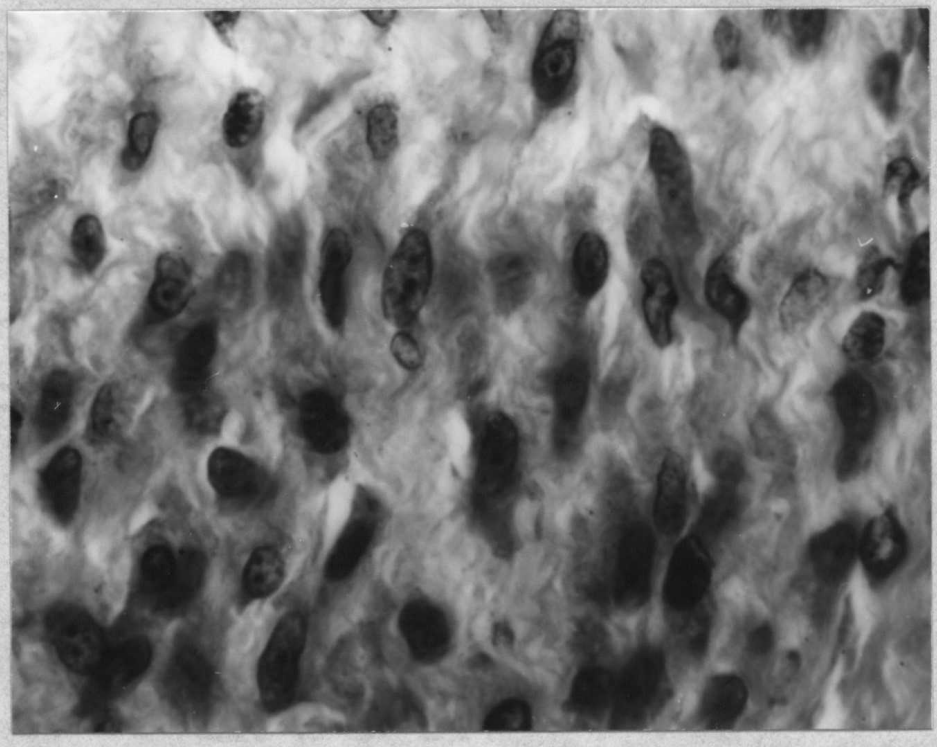

FIGURE 21

PLATE X

PLATE XI

Figure 22. Photomicrograph of the inner layer of periosteum and bone at the insertion of the pectineus and adductor longus from a Group 1 rat. Note the osteoblasts on the surface of the bone. o, osteoblasts; b, bone; p, inner layer of periosteum. Masson's trichrome. X900.

Figure 23. Photomicrograph of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 1 week. al, adductor longus tendon; ph, pectineus; o, outer layer of periosteum; i, inner layer of periosteum; b, new bone. Masson's trichrome. X47.

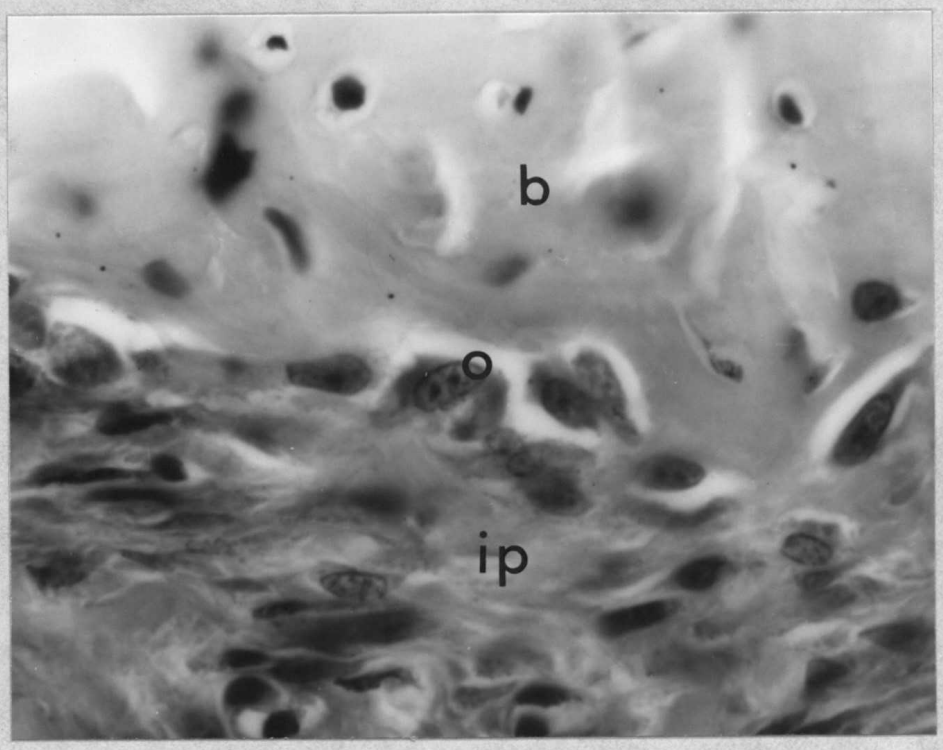

FIGURE 22

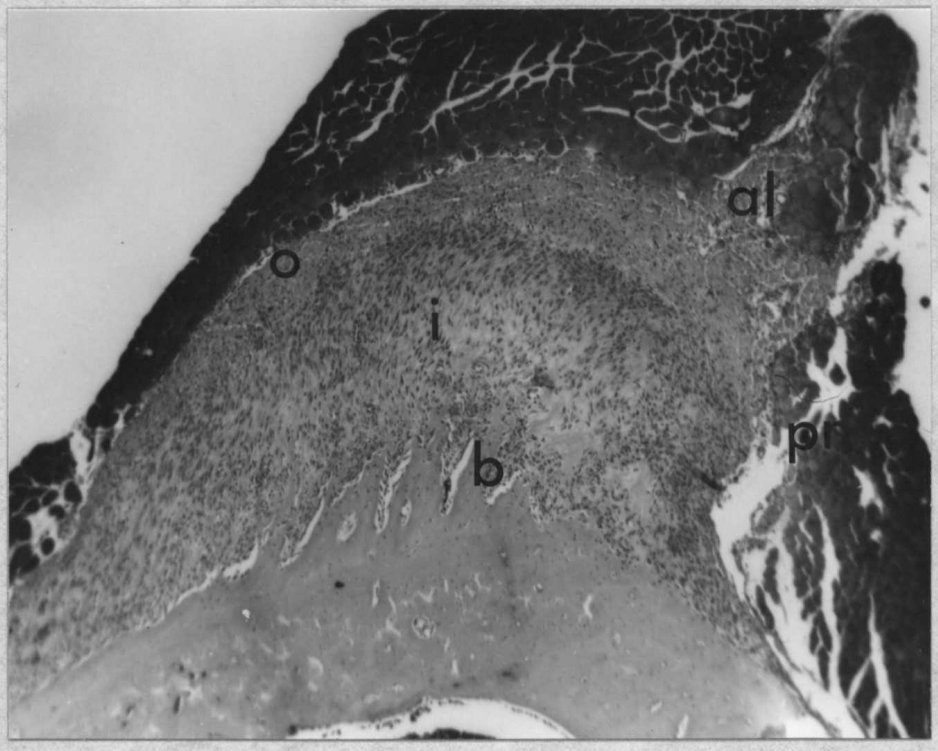

FIGURE 23

PLATE XI

PLATE XII

Figure 24. Photomicrograph of the inner periosteum of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 1 week. Note the enlarged nuclei of the periosteal cells and relatively small amount of connective tissue. Masson's trichrome. X900.

Figure 25. Photomicrograph of the intercellular zone of the inner layer of periosteum of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 1 week. Note the large amount of intercellular material. Masson's trichrome. X900.

FIGURE 24

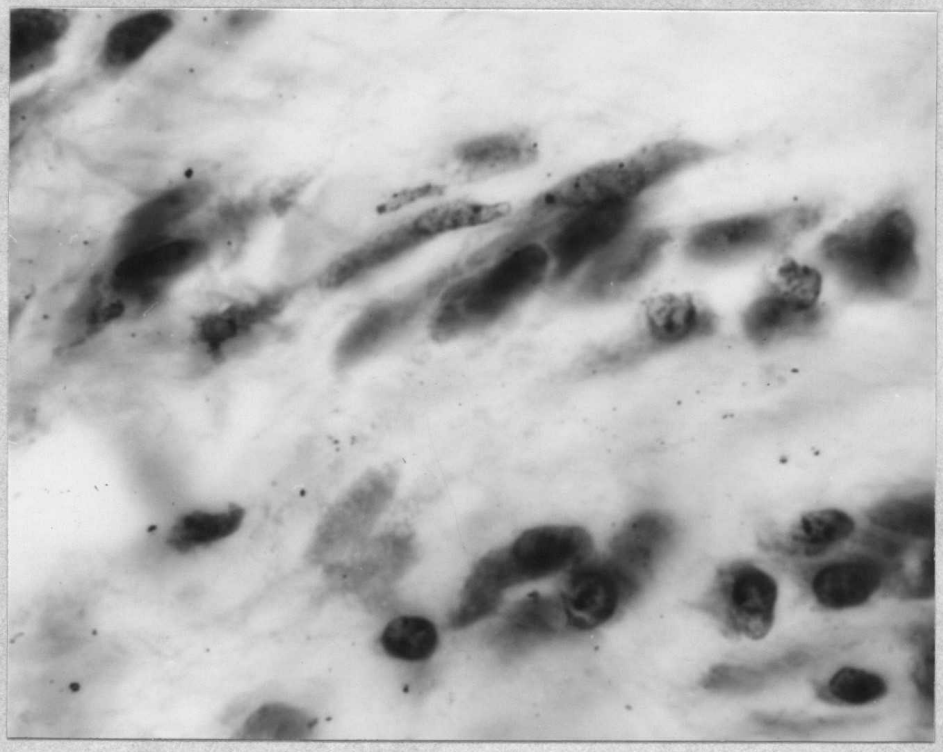

FIGURE 25

PLATE XII

PLATE XIII

Figure 26. Photomicrograph of the osteogenic zone of the inner periosteum of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 1 week. b, new bone; o, osteoblast; m, premarrow; v, blood vessel. Masson's trichrome. X520.

Figure 27. Photomicrograph of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 3 weeks. Note the large amount of newly formed bone. b, new bone; f, fibrous portion of the exostosis. Masson's trichrome. X47.

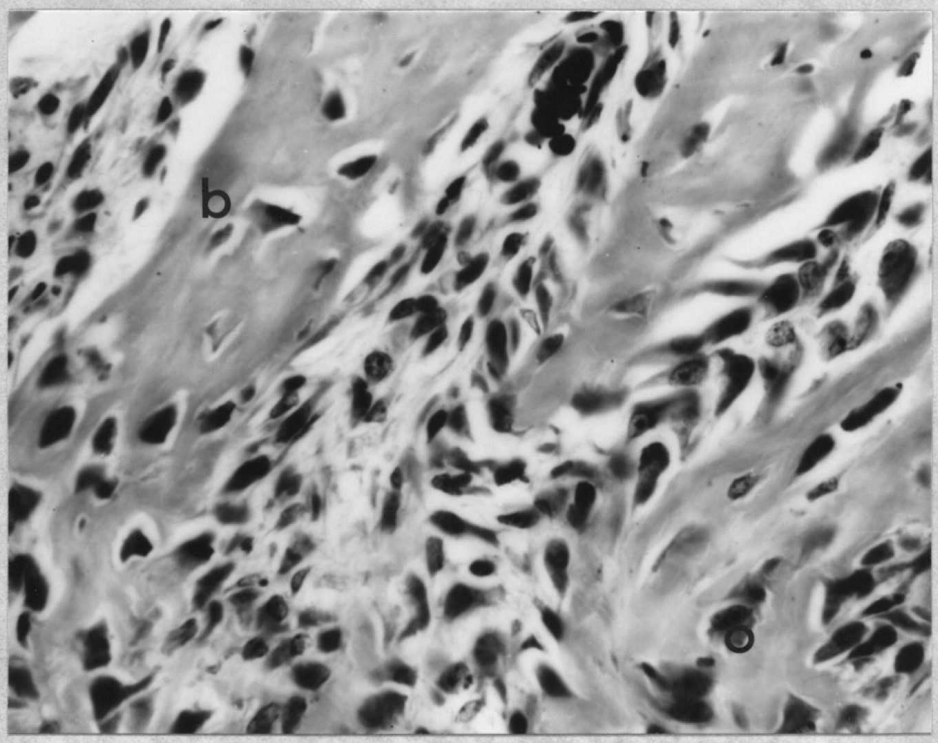

FIGURE 26

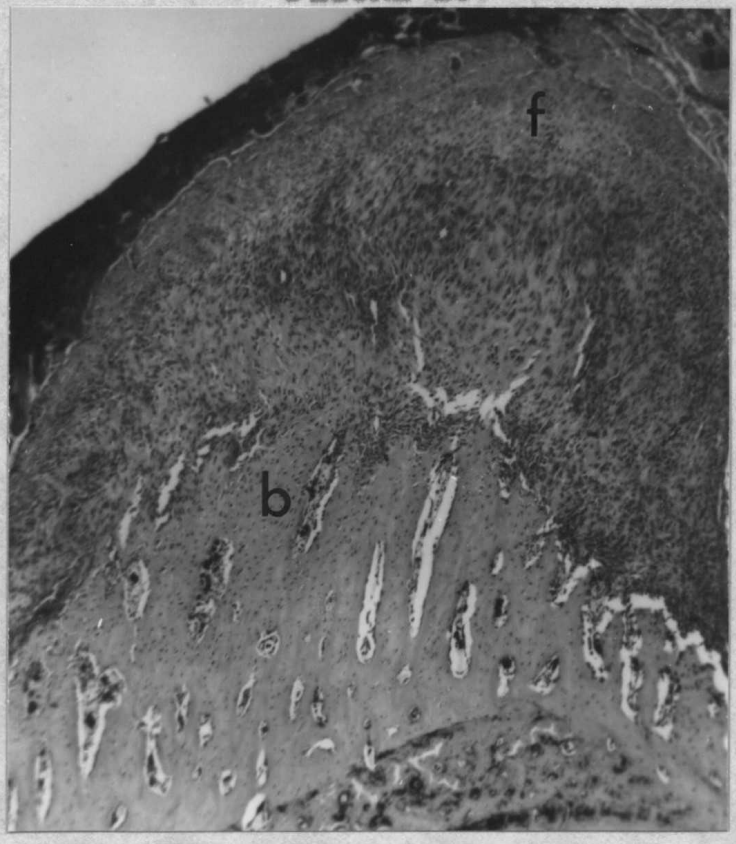

FIGURE 27

PLATE XIII

PLATE XIV

Figure 28. Photomicrograph of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 5 weeks. b, bone in exostosis; ct, fibrous protion of exostosis; m, marrow space. Masson's trichrome. X47.

Figure 29. Photomicrograph of an area of cartilage in a pectineus-adductor longus exostosis from a Group 4 rat given BAPN for 4 weeks. c, cartilage cell; x, cartilage matrix. Masson's trichrome. X900.

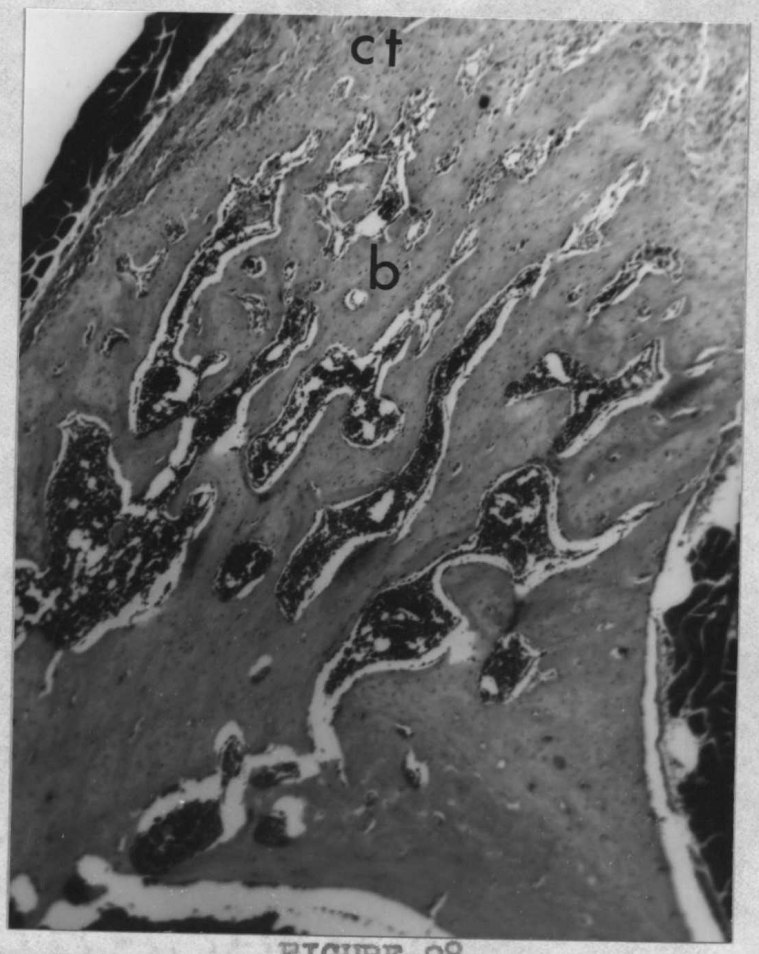

FIGURE 28

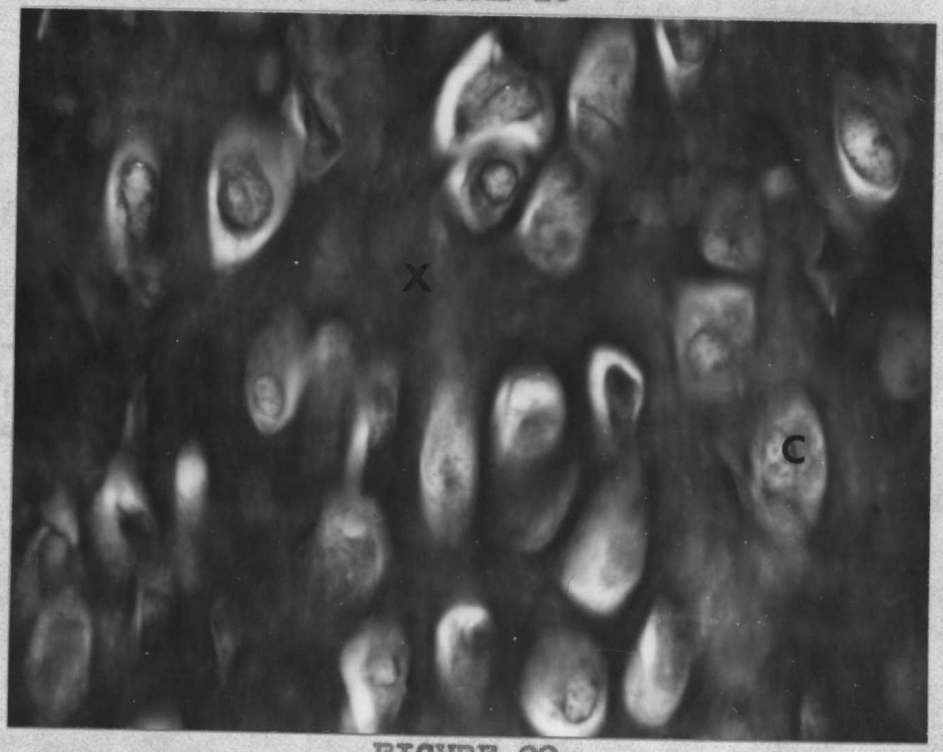

FIGURE 29

PLATE XIV

PLATE XV

Figure 30. Photomicrograph of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 5 weeks. Note the dark areas which have stained metachromatically. c, cartilage; b, bone; fibrous tissue, Azure A. X118.

Figure 31. Photomicrograph of a tip of a bone spicule containing cartilage in a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 6 weeks. c, cartilage; Masson's trichrome. X205.

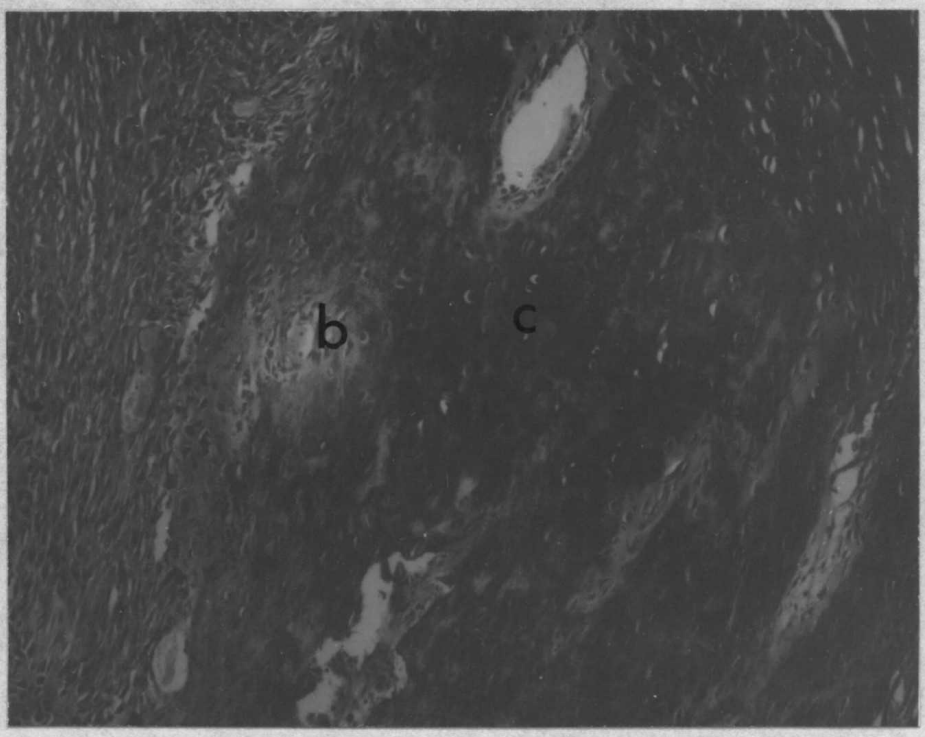

FIGURE 30

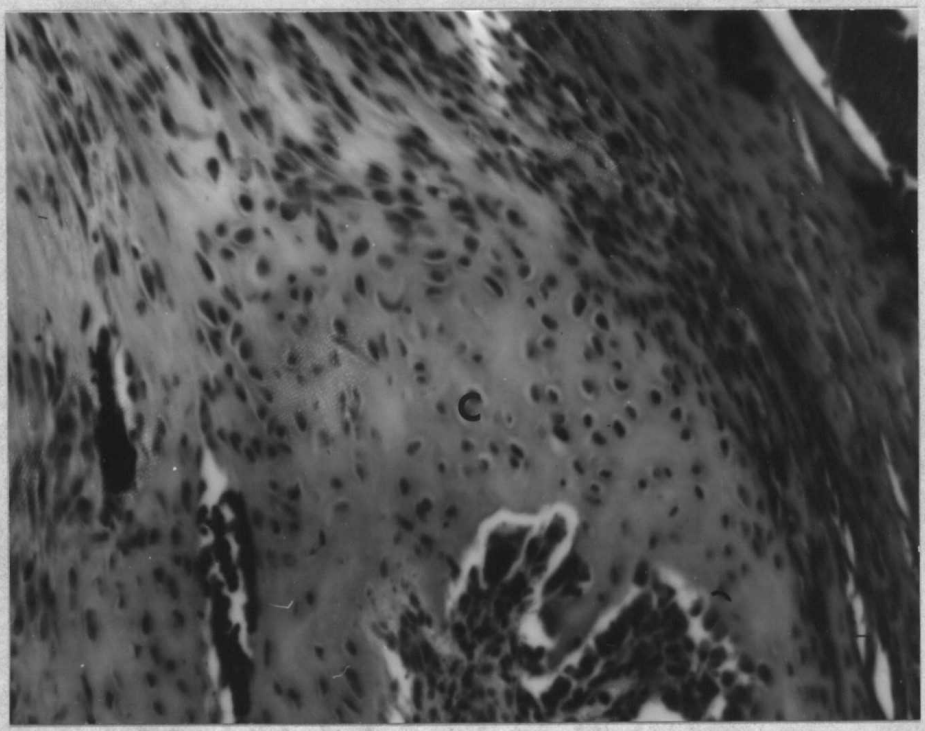

FIGURE 31

PLATE XV

PLATE XVI

Figure 32. Photomicrograph of an area of a bone spicule in a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 3 weeks. g, basophilic globule; o, lacuna with osteocyte. Hematoxylin and eosin. X2100.

Figure 33. Photomicrograph of an area of bone within a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 5 weeks. g, metachromatic globules; o, lacuna with osteocyte. Azure A. X520.

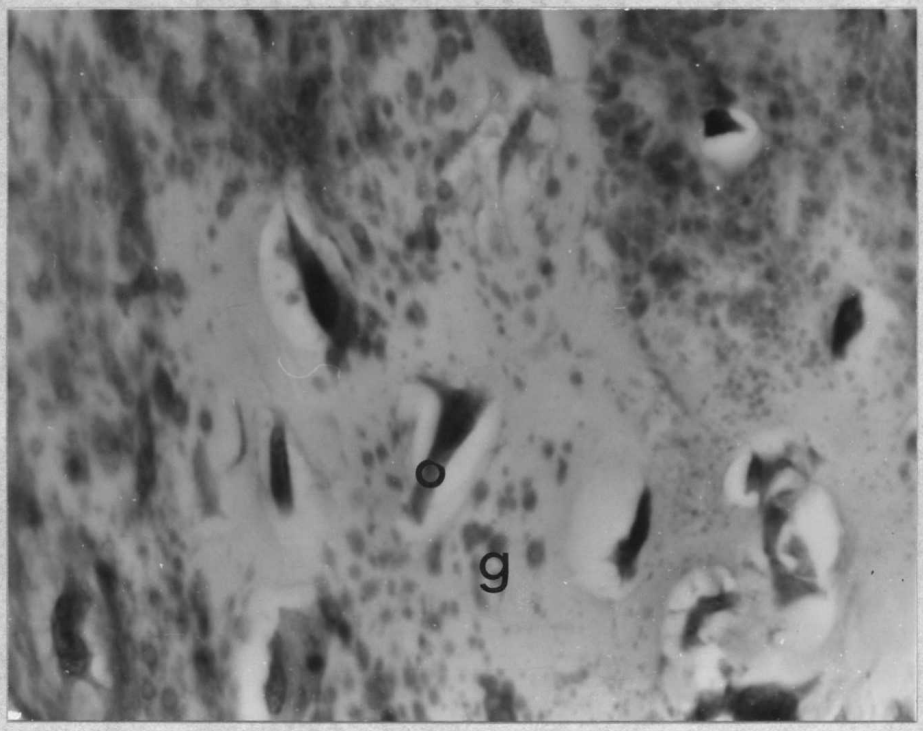

FIGURE 32

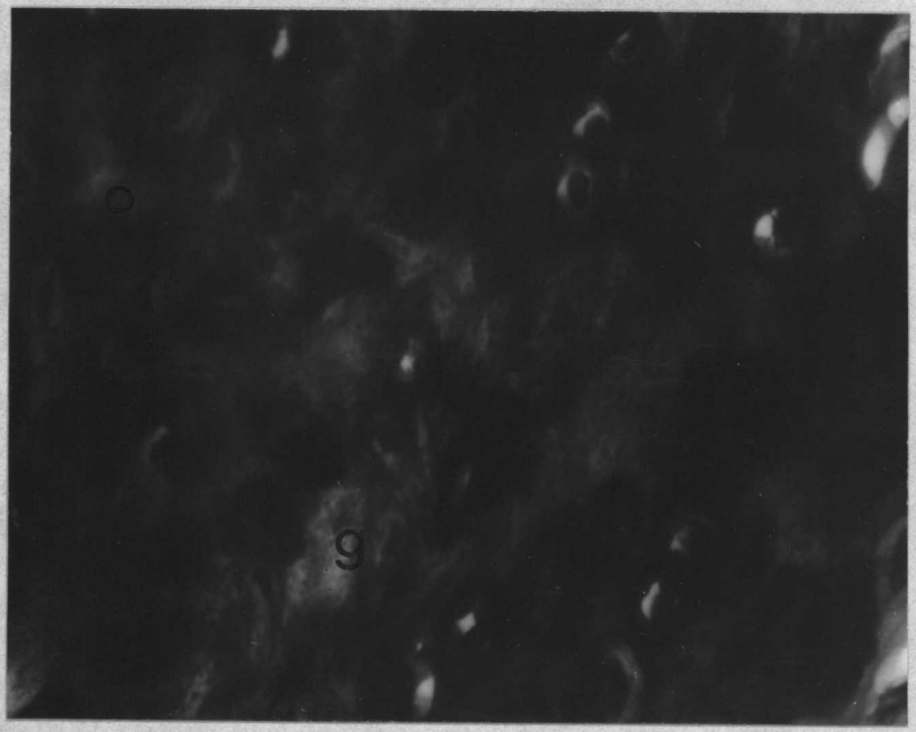

FIGURE 33

PLATE XVI

PLATE XVII

Figure 34. Photomicrograph of a pectineus-adductor longus exostosis from a Group 4 rat given BAPN for 4 weeks. Note the large area of newly formed bone. Masson's trichrome. X47.

Figure 35. Photomicrograph of a pectineus-adductor longus exostosis from a Group 4 rat given BAPN for 5 weeks. Note size compared to Figure 36 which is the same magnification. Masson's trichrome. X47.

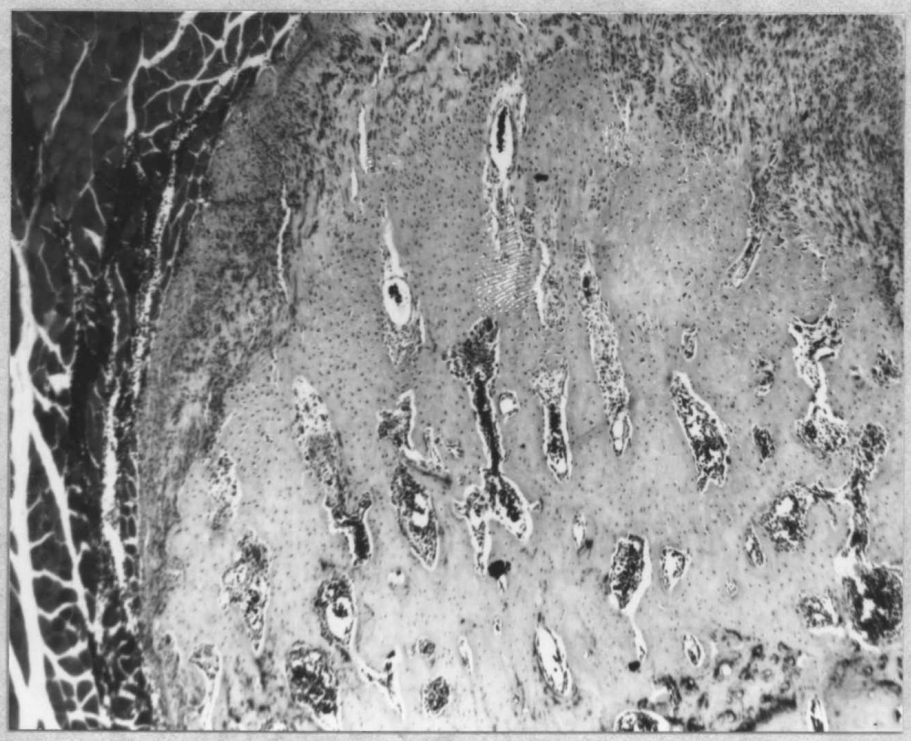

FIGURE 34

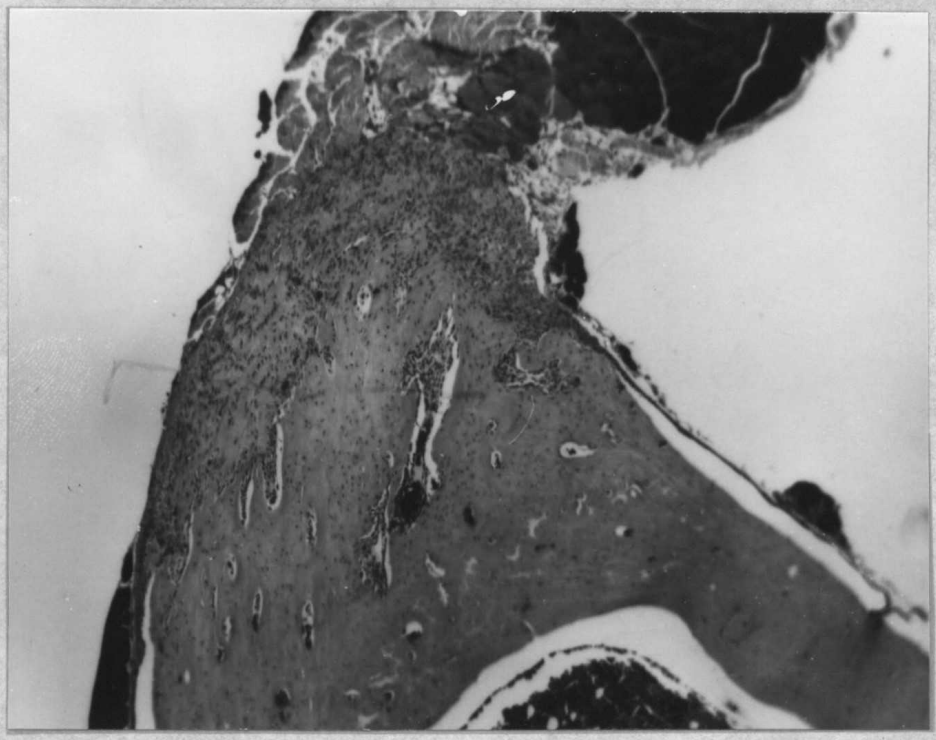

FIGURE 35

PLATE XVII

PLATE XVIII

Figure 36. Photomicrograph of a pectineus-adductor longus exostosis from a Group 2 rat given BAPN for 5 weeks. Note the large marrow spaces and the extent of the bone formation on each side. m, marrow space. Masson's trichrome. X47.

Figure 37. Photomicrograph of a pectineus-adductor longus exostosis from a Group 6 rat given BAPN for 6 weeks. b, bone; ct, connective tissue. Masson's trichrome. X47.

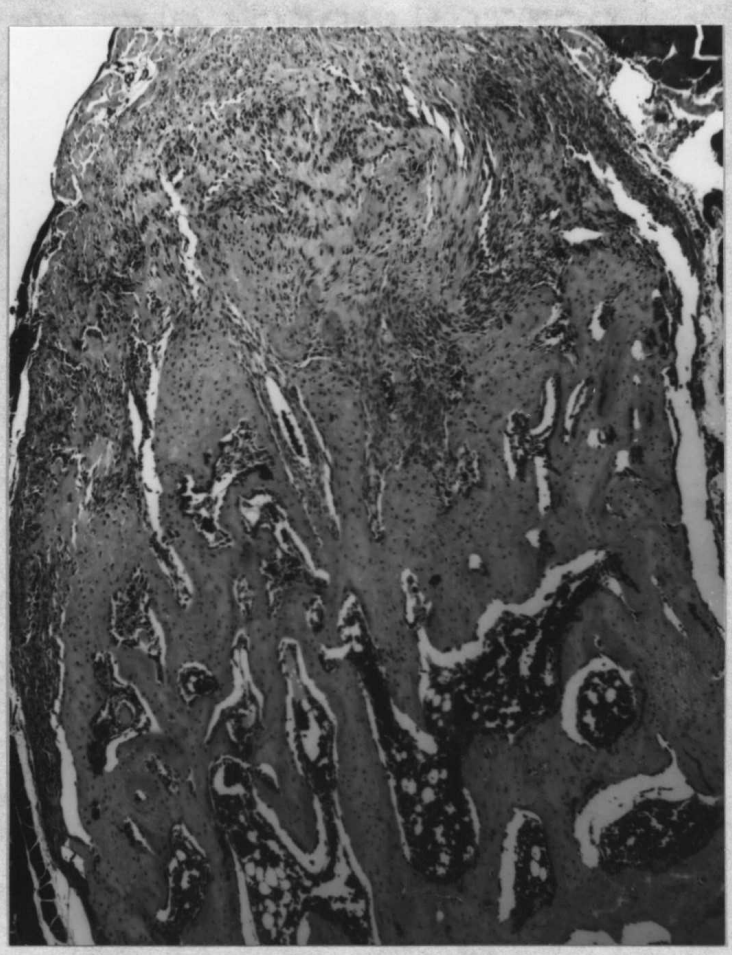

FIGURE 36

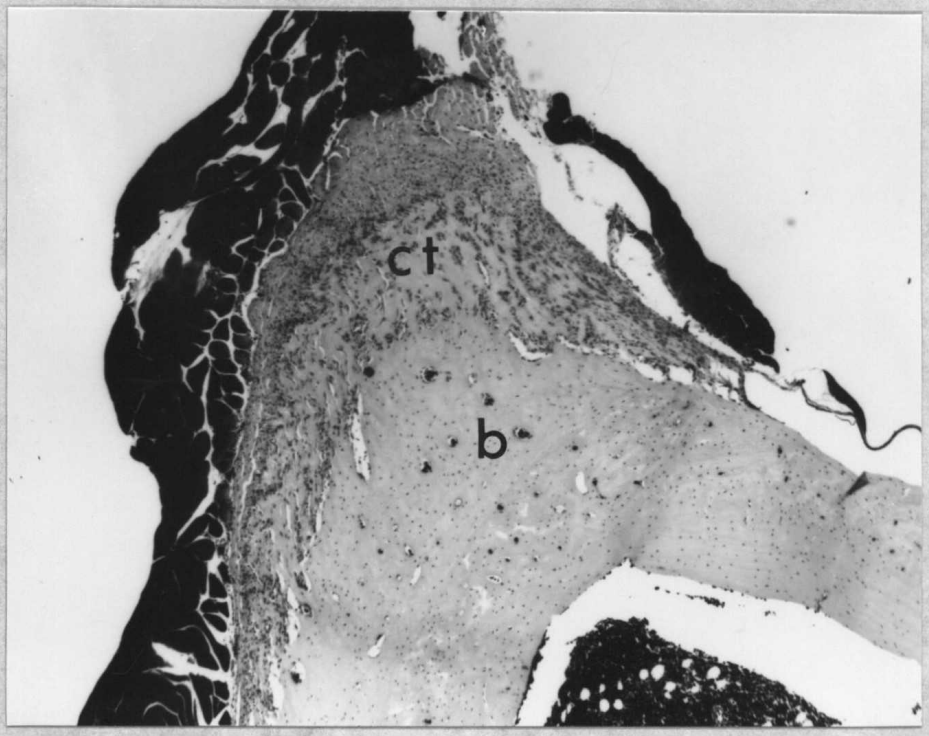

FIGURE 37

PLATE XVIII

PLATE XIX

Figure 38. Photomicrograph of an area of the connective tissue in a pectineus-adductor longus exostosis from a Group 6 rat given BAPN for 6 weeks. t, fibroblast; c, collagen bundle. Masson's trichrome. X520.

Figure 39. Photomicrograph of an area peripheral to the bone in a pectineus-adductor longus exostosis from a Group 6 rat given BAPN for 5 weeks. t, fibroblast; p, periosteal cell; o, prebone area. Hematoxylin and eosin. X520.

FIGURE 38

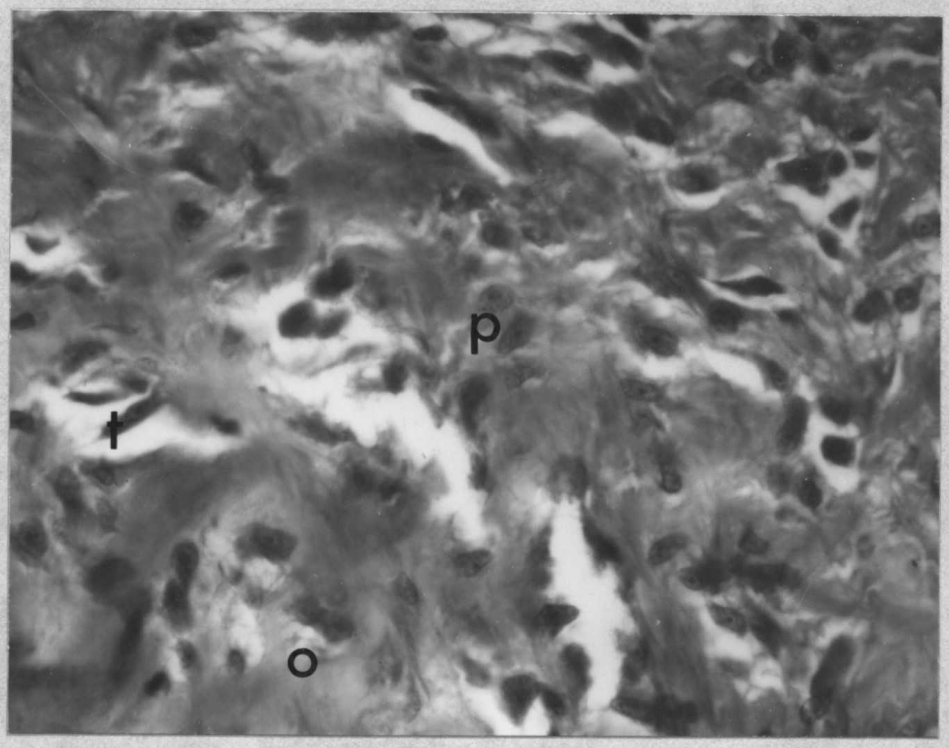

FIGURE 39

PLATE XIX

PLATE XX

Figure 40. Photomicrograph of a pectineus-adductor longus exostosis from a Group 6 rat given BAPN for 5 weeks. Note the cross-sectional shape of the bone within the exostosis and compare with Figure 36. Hematoxylin and eosin. X47.

Figure 41. Photomicrograph of a pectineus-adductor longus exostosis from a Group 6 rat given BAPN for 5 weeks. Note the small marrow spaces. m, marrow space; b, bone; Masson's trichrome. X118.

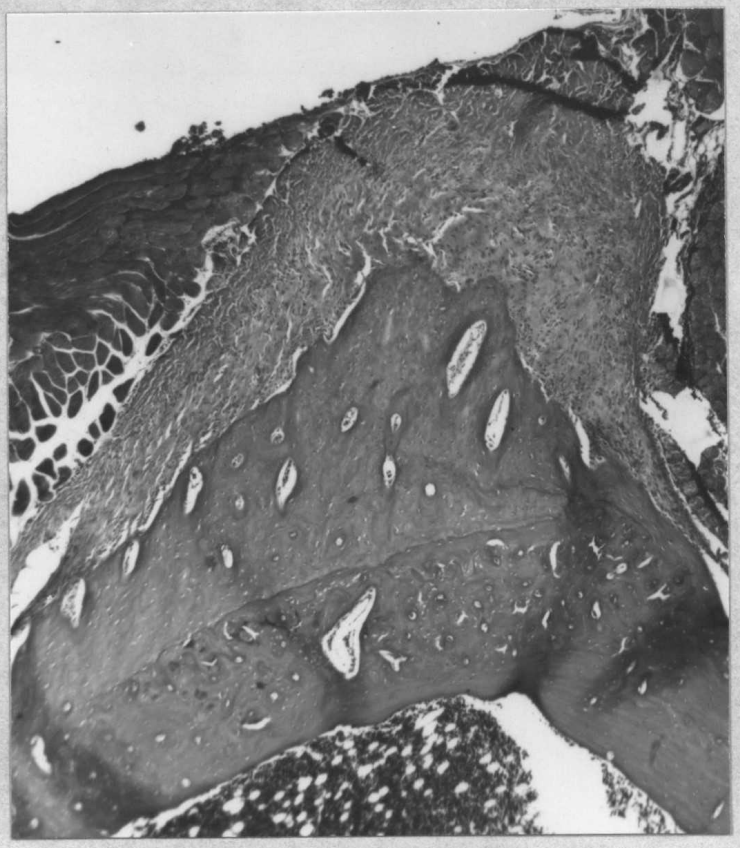

FIGURE 40

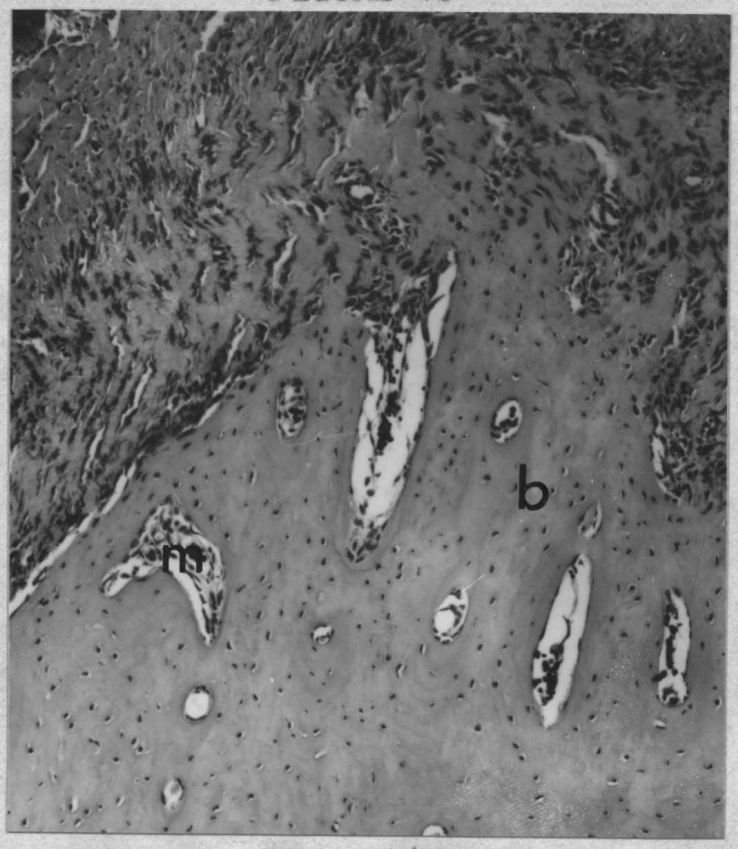

FIGURE 41

PLATE XX