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AN AUTORADIOGRAPHIC STUDY OF THE EFFECT OF
BETA-AMINOPROPIONITRILE ON THE
PRELABELED MATURE COLLAGEN
OF THE PREPARTUM
RAT UTERUS

by

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B.S. in Biology, Wisconsin State University,
River Falls, 1965

A Thesis
Submitted to the Faculty
of the
University of North Dakota
in partial fulfillment of the requirements
for the Degree of
Master of Science

Grand Forks, North Dakota

August
1967

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This thesis submitted by James E. Ingli in partial fulfillment of the requirements for the Degree of Master of Science in the University of North Dakota is hereby approved by the Committee under whom the work has been done.

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253388

ACKNOWLEDGEMENTS

The author would like to express sincere appreciation to Dr. Vernon L. Yeager for his immeasurable advice and encouragement in the development of this research.

Deep gratitude is expressed to Dr. Dwayne A. Ollerich for his constructive criticism and assistance in the preparation of this paper.

Special recognition goes to Mr. Kenneth Hardy and Mr. Michael Snodgrass for the help in the preparation of the figures.

Sincere thanks are also expressed to Dr. Theodore Snook and Dr. Helge Ederstrom for their criticism and help in the preparation of this paper.

The author acknowledges support from Traineeship 5TI-GM-1014, National Institutes of Health, United States Public Health Service for this research.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
LIST OF TABLES AND GRAPHS	v
LIST OF PLATES AND FIGURES	vi
ABSTRACT	viii
Chapter	
I. INTRODUCTION	1
II. MATERIALS AND METHODS	15
III. RESULTS	18
Gross Observations Histological Observations Autoradiographic Observations	
IV. DISCUSSION	29
V. SUMMARY AND CONCLUSIONS	36
APPENDIX I	40
APPENDIX II	59
LITERATURE CITED	61

LIST OF TABLES AND GRAPHS

Page

TABLE 1 24

Percentage variation of the average counts per 0.06 μ^2 for extracted control biopsies when compared to respective nonextracted biopsies.

TABLE 2 25

Percentage variation of the average silver grain counts per 0.06 μ^2 for saline extracted experimental biopsies when compared to respective nonextracted biopsies.

TABLE 3 26

Comparison of average silver grain counts per 0.06 μ^2 for each biopsy.

TABLE 4 60

"T" test of significance of nonextracted and extracted experimental average silver grain counts per biopsy.

GRAPH 1 27

Bar graph of silver grain counts for the first and second biopsies of each control animal.

GRAPH 2 27

Bar graph of silver grain counts for first and second biopsies of each experimental animal.

GRAPH 3 28

Bar graph of silver grain counts for the third biopsy each of control and experimental animal.

LIST OF PLATES AND FIGURES

Page

PLATE I: 41

Figure:
1. Photomicrograph of longitudinal section of normal rat uterus adjacent to implantation site.

PLATE II:. 43

Figure:
2. Photomicrograph of mucosa from nonextracted control showing epithelium and adjacent connective tissue.
3. Photomicrograph of mucosa of nonextracted control showing PAS positive film on the luminal surface of epithelium and fibrillar incomplete basement membrane.

PLATE III: 45

Figure:
4. Photomicrograph of mucosa of nonextracted control showing concentration of reticular fibers near the base of the epithelial cells.
5. Photomicrograph of mucosa of nonextracted control showing cells and vessels in subepithelial connective tissue.

PLATE IV:. 47

Figure:
6. Photomicrograph of a vessel from a nonextracted control showing PAS positivity in the vascular smooth muscle.
7. Photomicrograph of myometrium of nonextracted control showing reticular fibers and nets.

PLATE V:

Figure:
8. Photomicrograph of longitudinal section of nonextracted control showing PAS positive reaction in the myometrium.

9. Photomicrograph of mucosa and circular layer of myometrium from control tissue showing effects of saline extraction.

PLATE VI: 51

Figure:
10. Photomicrograph of myometrium from extracted control showing reticular arrangement after extraction.
11. Photomicrograph of myometrium from extracted control showing effect of saline extraction on PAS reaction in smooth muscle.

PLATE VII: 53

Figure:
12. Photomicrograph of mucosa from extracted BAPN treated tissue showing fraying of epithelium and removal of PAS positive film.
13. Photomicrograph of saline extracted BAPN treated tissue showing disruption of the reticular fiber and net arrangement in longitudinal layer of myometrium.

PLATE VIII: 55

Figure:
14. Photomicrograph of section of uterine tissue from third biopsy of BAPN treated animal showing blood vessel and concentration of leucocytes in the interstitial spaces.
15. Photomicrograph of an autoradiograph of non-extracted control tissue showing silver grain distribution of mucosa.

PLATE IX: 57

Figure:
16. Photomicrograph of an autoradiograph of non-extracted control tissue showing silver grain distribution of circular layer of myometrium.
17. Photomicrograph of an autoradiograph of extracted experimental tissue showing increased tissue spaces and cell shrinkage in longitudinal myometrial layer.

ABSTRACT

Maturation of the collagen fiber involves aggregation of the tropocollagen molecule through increasing numbers of inter- and intramolecular crosslinkages. Maturation is reflected in the transition of soluble to insoluble collagen. An experimental model, lathyrism, induced by beta-aminopropionitrile (BAPN), alters the normal collagen bonding and produces an increase of the saline-soluble collagen. Conflicting reports have been made concerning bond alteration of the insoluble fiber by BAPN, reverting the collagen to a more soluble state. The prepartum rat uterus, with collagen of a high turnover or maturation rate, was used to determine the contribution of the insoluble collagen fiber to the lathyric collagen.

Ten albino rats were injected with the labeled amino acid L-proline-H^3 on the seventeenth day of gestation to label the collagen of the uterus. Thirty-six hours later half the animals were injected with BAPN while the other half served as controls. Biopsies of the uterine horns were taken from anesthetized rats at two, three and four hours post-BAPN injection. Tissue from one horn was fixed immediately in buffered neutral 10% formalin while that from the other horn was extracted with cold 1 M saline for 24 hours

prior to fixation. The effect of cold saline on the uterine-insoluble collagen was compared in control and BAPN injected groups using histological stains and autoradiograph techniques.

Microscopic evidence of tissue shrinkage caused by hypertonic 1 M saline extraction was noted. It was felt that this shrinkage was responsible for the high silver grain counts noted in most autoradiographs of extracted tissue. The cytoplasm of the mucosal epithelium of BAPN-treated tissue was more acidophilic than the control tissue. Disruption of reticular fibers and nets was noted in saline extracted experimental tissue when compared to extracted control tissue.

A decrease in the average silver grain counts was noted in non-extracted and extracted experimental tissue when compared to control tissue. This finding suggested a possible action of BAPN on uterine collagen before the first biopsy was taken. However, no evidence was obtained suggesting that BAPN caused the mature collagen of the prepartum rat uterus to revert to a saline-soluble form.

CHAPTER I

INTRODUCTION

The basic materials responsible for maintaining the morphological integrity of the vertebrate body are the connective tissues. Histologically, adult connective tissue has been divided into three main groups--the connective tissue proper, bone and cartilage (1). Collectively, these substances render the otherwise soft tissue of the vertebrate body rigid giving the internal organs the ability to stretch, expand and return to their original shape and to provide external stability important for locomotion, protection and the manual functions of the specific animal. The connective tissue proper also serves as a "packing material" between the histological components of the various organs. Each of the connective tissues is composed of cells and intercellular material.

Cells commonly associated with the connective tissues can be considered in two categories, a "transient" and two type of "stationary" varieties. Those considered "transient" pass through the connective tissue proper during various activity states. In this category are macrophages, lymphoid wandering cells and eosinophils. Two types of "stationary" cells are associated with connective tissue. Mast cells, pigment cells and fat cells are connective tissue specific, but

can be absent entirely from the tissue. Where variability exists in the first group, the second group of "stationary" cells is always found in the connective tissue. These cells are fibroblasts associated with the connective tissue proper, osteoblasts and osteocytes associated with bone, and chondroblasts and chondrocytes associated with cartilage. The fibroblast is generally believed to form the precursors of the collagen fiber in the soft connective tissues, while the chondroblast and osteoblast form the collagen fiber elements of cartilage and bone. The chondroblast, fibroblast and osteoblast have their origin in the embryo from the undifferentiated mesenchymal cell. Later the chondroblast and osteoblast are derived from the young fibroblast in the perichondrium and periosteum by cyto-differentiation (1).

Fibroblasts are long, flat elements appearing spindle-shaped in profile (1). Their elongated or star-shaped bodies send out several spear-shaped processes ending in one or several points. The cell body is demonstrable with iron hematoxylin. The cell has an oval nucleus (sometimes a slightly folded outline) which contains dust-like chromatin particles and one or more large nucleoli (1). Rough surfaced endoplasmic reticulum and free ribosomes are components of the cytoplasm and are found in quantity in active fibroblasts (2). The prominence of rough surfaced endoplasmic reticulum is evidence of secretory properties inherent in fibroblasts (2). Adjacent to the nucleus are a diplosome and Golgi apparatus (1). Mitochondria, appearing as slender rods, are scarce in

the processes but numerous near the nucleus and around the cytocentrum. The fibroblast possesses motile properties, exhibited during inflamation of tissue when it moves with a gliding motion with no ameboid pseudopodia observable (1).

The cells associated with the connective tissues are held in a self-derived intercellular material which consists of a ground substance and various fibrous proteins (3).

The ground substance appears to be a homogenous material defined as that portion of the connective tissue exclusive of the cellular and fibrous components (1). It is the "milieu interieur" through which nutrients, metabolites, ions and water pass to all cells of an organism (3). The ground substance contains in part the secretions of the fibroblast from which are formed the fibrous elements (3). It also contains various acid mucopolysaccharides among which are hyaluronic acid, chondroitin sulfate A, B and C, chondroitin, keratosulfate and heparin sulfate (3). Other constituents present with greater variation are proteins, soluble collagens, water, enzymes, hormones, vitamins, ions, cellular metabolites, albumen, and globulins (3). Alterations of the ground substance have been described with aging, variations in activity of the tissue and pathological disorders of the connective tissues (3). It is affected by hormones such as relaxin, adrenocorticotrophic hormone and cortisone (1). It is implicated in processes of bone and cartilage formation, resorption and calcification (1).

The quantity of ground substance in any one type of connective tissue is dependent upon the arrangement and density of the fibrous elements present. In bone tissue there is a minimal amount of ground substance squeezed between the tightly-packed, longitudinally-oriented collagen fibers (3). The ground substance consists of large quantities of the bone mineral hydroxyapetite, which contains the minerals calcium and phosphate in high concentrations (3). In hyaline cartilage the ground substance exhibits marked basophilia (1). This property is contributed by chondromucoid, a glycoprotein which on hydrolysis yields chondroitin sulfate (1). In this case the ground substance is composed of approximately one-third chondroitin sulfate (1).

The other constituents contributing to the strength and form of the connective tissue are the fibrous elements. The three fibrous elements are elastic fibers, reticular fibers and collagen fibers.

The term "elastic fiber" describes its tensile qualities (4). It is found in quantity in forms of connective tissue that undergo stretching and contraction, such as the aorta and ligamentum nuchae (1). It is also found to a lesser degree in other forms of the connective tissue proper (1). The elastic fiber is a wholly insoluble, highly branched fiber consisting of polysaccharides containing large amounts of hexosamine and uronic acid in intimate association with an insoluble protein (4). This insoluble protein material does not contain the amino acid hydroxylysine and contains less than 37

percent hydroxyproline (4). Structurally, elastic fibers have been described as amorphous in nature (1). However, Taylor and Yeager (5) have observed ultrastructural filamentus subunits termed by them "elastofibrilli" in the elastic fiber of rat periosteum. These findings have not been confirmed by X-ray diffraction studies (1,4).

Reticular fibers occur in connective tissue as highly branched interwoven networks (1). These fibers usually occur where connective tissue is adjacent to other tissue (1). Most stains do not demonstrate reticular fibers, however, with modified Bielschowsky methods of silver impregnation (i.e., Snook's modification, 6) they appear as black, sharply drawn nets (1). In the basement membrane, they form a dense network separating the epithelium from the connective tissue (1). Networks of this fibrous element are formed around blood vessels, muscle fibers, nerve fibers, fat cells, and in the respiratory portions of the lung. Reticular fibers are found to be inelastic (1). They have similar physico-chemical properties to collagen and like collagen fibers, resist digestion by trypsin in an alkaline medium (4). Reticular fibers, by some authors, are considered to be a type of intermediate collagen fiber (1). However, unlike collagen fibers and perhaps owing to their simpler nature, they do not swell in solutions of dilute organic acids (4).

The third fibrous constituent of connective tissue is the collagen fiber. Collagen is characterized by an unusual amino acid composition. Approximately one-third of the amino

acid residues are glycine and about one-fourth are proline and hydroxyproline (7, 8). Other than these extremely abundant amino acids, the basic collagen precursor (termed procollagen) is made up of an assortment of amino acids. Unlike the "ordinary" proteins, which after end-to-end linkages have been made remain unaltered, collagen synthesis includes alterations of the amino acids lysine and proline after the procollagen-polypeptide chain has formed (8). The mechanism of alteration has been shown to be an aerobic hydroxylation of these amino acids to form hydroxylysine and hydroxyproline (9). Cooper and Prockop (9) have shown, in embryonic chondroblasts, that this hydroxylation takes place while the procollagen is still in the cytoplasm of the cell and only after aerobic hydroxylation is the procollagen element removed to the intercellular matrix.

Upon extrusion of procollagen from the cell, a process of intra- and intermolecular crosslinkages initiates formation of the basic collagen molecule referred to as tropocollagen (10). Tropocollagen is described by Piez (11) as a cylinder about 3000 Å long and 14 Å in diameter with a molecular weight of about 30,000. The molecule is three stranded, each strand being a modified polyproline helix which has a repeat distance of about 9 Å and a three amino acid repeat. The three strands are wound together in a rope-like fashion to produce a triple helix with a repeat order of 100 Å. Stabilization and rigidity of the molecule is accomplished by interchain hydrogen bonds and steriochemical restrictions supplied by the pyrrolidine rings of proline and hydroxyproline. This represents a tightly

packed structure with the triplet of Glycine-x-y (11). Glycine residues occur at every third position and hydroxyproline invariable occurs at the Y position with proline at either X or Y (10). The X and Y positions can be occupied by any of the amino acids, but hydroxyproline and proline occupy them most frequently as indicated by the high content of these residues available on analysis (11).

The tropocollagen molecules aggregate through intermolecular and intramolecular linkages to form the collagen fibril (11). The molecules aggregate with their axes parallel but overlapping in a regular fashion to produce a major repeat of 640 A, the typical banding which is seen with the electron microscope (11). The long term stability inherent to the collagen fiber does not take place until covalent crosslinkage produces stabilization of the collagen fibril (11,12). Stabilization by crosslinkage has been shown to be the process by which the collagen fibril matures and is related to variations in collagen extraction by saline, acetic acid and guanidine (13). Designation of the chain units obtained from collagen have been made according to the number of polypeptide chains banded together. A single polypeptide chain has been designated as the alpha component. A dimer of the alpha chain has been designated as the beta component and an alpha chain trimer has been designated as the gamma component. Extraction with cold saline yields a collagen fraction high in alpha and beta components and this fraction has been shown to represent newly synthesized collagen. Acetic acid extraction yields a

fraction which consists largely of beta components but does contain some alpha chains. The nature and role of acid soluble collagen are unclear, but it has been proposed that intramolecular linkages predominate in this fraction and hinder its capabilities to form intermolecular bonds (13). This would account for its slow conversion into insoluble collagen (14). Extraction with 5 M guanidine also yields a collagen fraction with a very high percentage of beta components, higher than could be expected from intramolecular-crosslinked collagen alone (13). This fraction apparently represents a collagen which is partly intermolecularly bonded and that is further along the maturation scale. Extraction with strongly acidic buffers yields a preponderance of beta components which most likely would be intermolecularly bonded (13). The final steps in the maturation process would be the conversion of these alpha and beta components to gamma components and higher aggregates (11, 13, 15).

After the bonding of these chains is complete and they are in their non-extractable or insoluble state, the completed collagen fiber appears relatively inert (16). However, collagen synthesis and turnover rates have been determined for various rat tissue (17). It was found that the synthesis and turnover rates varied with the type of tissue, i.e., aorta, uterus, tendon, and skin. In comparing these tissues with regard to descending amounts of synthesis taking place, the tissues ranked skin, aorta, and tendon. If ranked according to turnover rates it would be skin, tendon, and aorta. Comparing the

tissues studied, uterine tissue had the greatest synthesis and turnover rate.

Correlation of this study with another done by Harkness and Harkness (18) on collagen content of the uterus during pregnancy and lactation, describe the uterus as an organ of tremendous collagen synthesis and accummulation with a very rapid conversion of the soluble collagen to its insoluble mature form. The accummulation of collagen is accompanied by enlargement of smooth muscle cells and fibroblasts (1). However, according to other authors, an increase in the number of smooth muscle cells is due to cell division and transformation from undifferentiated cells in the area (1). Harkness and Harkness (18) recorded a slow increase in the collagen content of the uterine horns for the first ten days of gestation. Subsequently, the amount increased rapidly to almost four times its estrous value until parturition, after which rapid involution to near estrous value followed. The development of the uterine tissue is suggested to be influenced by the estrogen and progesterone produced by the ovary and in later gestation by the placenta (1, 16). It is thought the variation or withdrawal of these hormonal influences and/or the release of catabolic enzymes bring about the rapid involution noted complete within five days post-partum (18).

It is apparent that a tissue and its component parts can be influenced to extreme degrees by the absence or presence of a hormone, enzyme or chemical released in the ground substance of the tissue. Pathological changes also have been

induced in tissues by subjection to a foreign chemical in the ground substance. In 1933, Geiger _et al_. (20) demonstrated that rats fed a diet containing the seed of _Lathyrus odoratus_ developed lesions of the connective tissue. They described exostoses of bone at the point of tendonous muscle insertions, dissecting aortic aneurisms, kyphoscoliosis, and loss of tensile strength of connective tissue. This pathological change was termed lathyrism after the legume which induced it (21). Later, the disease in rats was referred to as osteolathyrism, differentiating it from neurolathyrism, a condition in humans caused by the ingestion of seeds from plants of the genus _Lathyrus_, which was manifested as a neurological disorder. In this paper the term "lathyrism" will denote the pathology known as osteolathyrism involving experimental animals. It was noted that lathyrism caused characteristics similar to several human disorders, such as the Marfan syndrome and arthroschlerosis (21). It was thought that by studying lathyrism insight into the cause and cure of the human disorders could be made.

The offending agent isolated from _Lathyrus odoratus_ was found to be a nitrile compound, beta-aminopropionitrile (BAPN) (21). While various other nitriles and chemicals were also found to produce similar lesions in laboratory animals, beta-aminopropionitrile has been the major lathyrogen used for experimentation.

Early investigators attempted to discover curative and protective agents to be used against lathyrism. However,

attempts at increasing various dietary nutrients failed to produce complete or even partial protection (23). Selye (24) initially reported a failure in attempts made to induce lathyrism in pregnant rats. He later showed an inhibition of lathyrogenesis by treatment with various hormones such as estradiol and somatotrophic hormone (STH) (22). It should be noted that he reported only the absence of gross lathyric changes due to the lack of information concerning the molecular changes discovered later. Subsequently, in the case of the pregnant rat, it was found that fetal resorption and uterine involution were gross changes caused by lathyrogens (24).

Simmons et al. (25) demonstrated that the normal endosteal bone formation produced by estrogen treatment of mice was greatly inhibited by subsequent or simultaneous treatment with the lathyrogenic agent amino acetonitrile (AAN). They also found that an increase in dosage of this lathyrogenic agent almost negated the effect of estrogen, thus suggesting that the previously inhibitory effect of estrogen was only temporary and dose dependent upon the lathyrogenic agent.

Another factor studied which was found to limit lathyrogenesis was the age of the animal (27). Young animals were found to be affected within hours or days by treatment with a lathyrogenic agent, while lathyric characteristics were found in older animals only after several weeks of treatment (27).

While these protective agents were being researched, attempts were made to determine the actual site of action of the lathyrogenic agent. The discovery of a profound involve-

ment of the connective tissues led to studies of the ground substance as a possible site of action. It was thought that the lathyrogenic agent in some way altered the ground substance such that the developing fibrous components failed to attain stability (21). The lack of stabilization of the fibrous components was proposed later to manifest itself at the level of the gross morphological characteristics seen in the lathyric animal. More recent studies have refined this proposal and presented data concerning the characteristics of the connective tissue components; mainly the collagen fiber with regard to characteristics of lathyric collagen.

Physico-chemical studies of the extractable collagen from lathyric animals indicated normal molecular dimensions, helical conformation and stability to thermal denaturation (28). Three abnormal properties were found to be characteristic of lathyric collagen: (1) lathyric collagen fibrils whether formed in vivo or in vitro were temperature sensitive and go back into solution upon cooling (28); (2) intramolecular crosslinking (involving beta subunits), a characteristic feature of normal acid extractable collagen, was markedly diminished in lathyric acid extractable collagen (29); (3) lathyric collagen was found unable to form stable intermolecular aggregates (30).

It is interesting to note that a doubling of the salt-extractable collagen was found to occur within two hours after BAPN administration (29). A change in the rate of collagen accummulation was not evident until forty-eight hours after the

single dose was administered and only then did it decrease (29). It was thought that the increased extractable collagen was derived from pre-existing mature (insoluble) fibrils (31, 32). That is, a disruption of insoluble collagen bonds by BAPN, or a factor produced in response to BAPN made the collagen susceptible to extraction. Another view held that there existed an impairment of the maturation process producing insoluble collagen fibrils and thus resulted in a accumulation of newly-synthesized collagen molecules (33, 34, 35). Tanzer and Gross (29), using a double labeling technique, concluded that the source of the lathyric collagen was derived from both sources--newly synthesized and mature insoluble fibers (29). They proposed that the lathyric process affected collagen in all states of aggregation but in varying degrees. The lathyric defect appeared to be a deficiency in intramolecular crosslinks, as shown by inability to form these crosslinks when incubated _in vitro_ (30, 31). With the isolation of an aldehyde-containing peptide from ichthyocal collagen (35, 37) and from rat skin collagen (37), the aldehyde constituent was proposed to be the site of action of several lathyric reactions (38, 39, 40). It has been reaffirmed that aldehydes are important in crosslinking and maturation of collagen (41). However, the exact effect of BAPN or other lathyric agents on the linkages involved in the maturation of the collagen fibers is still a question (21, 41). The controversy also remains as to the exact source of the increased saline extractable collagen fraction (21).

It has been determined, therefore, that BAPN acts to alter the bonds involved in the maturation of the collagen fiber. The alteration of these bonds at the time of synthesis is generally accepted to be a source of the increased saline-extractable collagen. However, the contribution to the saline-extractable collagen by cleavage or alteration of the pre-formed mature collagen fiber is still subject to question. It was believed that the collagen of the prepartum rat uterus, having a high synthesis and turnover rate, could be used to gain insight into the problem. Administration of a large dose of BAPN was thought to be the key factor in producing molecular pathologies in the prepartum rat. It is, therefore, the purpose of this study to observe the effects of beta-aminopropionitrile (BAPN) on the insoluble mature collagen of the prepartum rat uterus.

CHAPTER II

MATERIALS AND METHODS

Ten sexually mature female albino rats of the Sprague-Dawley strain were placed with males for breeding. Vaginal saline lavages were inspected daily. Females showing sperm were isolated with the day of sperm appearance labeled as day zero of pregnancy. The animals were subsequently maintained on Purina Laboratory Chow with an adequate supply of tap water, and weight was recorded each day.

On the seventeenth day of pregnancy all animals received intraperitoneal injections containing 0.5 μc/gm body weight of L-Proline-H^3 (Specific activity of 292 mc/mM Nuclear-Chicago Corporation). Thirty-six hours later, half the animals received intraperitoneal injections containing 1 mg/gm body weight of beta-aminopropionitrile fumerate (BAPN-Aldrige Chemical Co., Inc.) and the other half, serving as controls, were given intraperitoneal injections of equal quantities of physiological saline. Two hours post-injection, the animals were anesthetized using methoxyfluorane (Penthrane - Abbott Co.) after the technique of Hagen and Hagen (42). Laparotomy was performed utilizing a midline incision to avoid excessive bleeding. Biopsies of the most distal implantation site in its entirety (uterus, placenta and developing fetus)

were taken from each uterine horn. The open end of each uterine horn was closed by a single suture, the laparotomy clipped shut and the animal allowed to revive. The surgical procedure was repeated each following hour through four hours post-BAPN injection. First and second biopsies were taken from all animals. However, three control animals were sacrificed after the second biopsy because of adverse reaction to surgery. A total of 40 biopsies was taken.

The fetus and placenta were removed from each specimen and the uterine tissue from the left horn was fixed immediately in 10% buffered neutral formalin. Tissue from the right uterine horn was subjected to cold 1 M saline extraction for 24 hours prior to fixation. Paraffin embedded tissue was cut at six microns and mounted on chemically cleaned glass slides. Tissue to be used for autoradiography was stained by the Periodic acid-Schiff reaction, subsequently dip-coated with NTB-3 Kodak emulsion (Kodak Company) and exposed for 28 days at 4° C.

After exposure, the emulsion was developed in full strength Dektol, rinsed in distilled water, fixed in hypo, and washed in tap water for two hours. All solutions used were kept at 18°C. Slides were then counterstained with Harris' Hematoxylin and coverslipped for microscopic observation. Silver grain counts were made on only tissue which exhibited a typical uterine histology by using an ocular grid to measure a tissue area of .06 μ^2. Two tissue sections representing opposite areas of the uterine wall were selected, excluding

the site of placental implantation. Background counts were made visually on the emulsion adjacent to the section and four grid fields were counted through the myometrium. Background was subtracted from tissue grain counts and the counts for each biopsy were averaged and compared.

Differential stains for histological comparison were used on other mounted representative sections. The stains included Bielschowsky's silver impregnation of reticular fibers (Snook's modification, 6); elastin stain specific for elastic fibers; hematoxylin-eosin for nuclei and cytoplasmic structures; periodic-acid-Schiff reaction for glycogen, mucin, reticulin, collagen, basement membranes and fibrin; a Periodic-acid-Schiff counterstained with alcian blue, and Masson's trichrome (Lillie's modification) designed for nuclei, cytoplasmic structures, and various fibrous connective tissue components.

CHAPTER III

RESULTS

I. GROSS OBSERVATIONS

Initially all animals appeared healthy and possessed no obvious signs of respiratory disease or other illness. Daily weight gains were consistant for all animals. Water and food consumption appeared normal. Animals demonstrated no apparent ill effects from the intraperitoneal injections of L-proline-H^3 or beta-aminopropionitrile. After the first biopsy, sluggishness was noted in all animals. All viscera appeared normal at autopsy.

II. HISTOLOGICAL OBSERVATIONS

A. Nonextracted Controls

Longitudinal sections of rat uterus adjacent to implantation sites demonstrated a mucosa or endometrium, a myometrium and a serosa or perimetrium (Figure 1).

The mucosa consisted of an epithelium and an adjacent connective tissue layer. The mucosa demonstrated irregular foldings or convolutions, sometimes resembling villi. The epithelium varied from simple squamous to a low columnar type, with basally located nuclei which appeared round to oval in shape. The cytoplasm of the epithelial cells appeared

moderately basophilic but free of demonstrable cytoplasmic structures. Adjacent cell boundaries appeared indistinct resembling a syncytium (Figure 2). The luminal surfaces were covered with a thin PAS-positive film and the PAS reaction demonstrated an incomplete and apparently frayed basement membrane (Figure 3). The basement membrane that was present appeared to be fibrillar in nature. The reticulin stain demonstrated a concentration of reticular fibers near the base of the epithelial cells (Figure 4). A loose connective tissue layer was present subjacent to the epithelium. This layer was loosely arranged containing fine collagen fibers, reticular fibers and elastic fibers dispersed in an abundant ground substance. Typical fibroblasts were common in this layer with various other cells dispersed throughout. A large number of blood vessels and lymphatics were also present. Blood vessels were observed immediately subjacent to the epithelium causing, in some instances, an outward bulging of the epithelium (Figure 5). All vessels appeared to lack a distinct basement membrane. A high PAS-positive granulation was observed in the cytoplasm of the vascular smooth muscle cells (Figure 6). Isolated smooth muscle cells surrounded with reticular nets were occasionally observed in the sub-epithelial connective tissue. In addition, round tubular epithelial structures resembling glands were present in this layer.

The myometrium was located immediately adjacent to the endometrium. It consisted basically of two layers of

smooth muscle, a wide convoluted inner layer of circularly oriented cells, and an outer flat layer of longitudinally oriented cells (Figure 1). A thin layer of longitudinally oriented cells was sometimes observed immediately adjacent to the mucosa with a circular arrangement occurring in the remainder of the layer. The smooth muscle cells of the myometrium were loosely arranged, with connective tissue consisting of collagen, elastic and reticular fibers interwoven between them. Each smooth muscle cell seemed swollen or enlarged and was surrounded individually by a reticular net. Each net in turn was connected by reticular fibers to adjacent nets (Figure 7). The cytoplasm of the myometrial smooth muscle cells demonstrated a high PAS-positive reaction with the circular layer staining more intensely than the longitudinal layer (Figure 8). Often a loose connective tissue layer could be observed between the two layers of the myometrium resembling the connective tissue of the mucosa (Figure 8). When this layer was absent the two muscle layers seemed to merge and the boundary between the two layers became indistinct. Blood vessels were found between the smooth muscle cells and in the connective tissue layer that separated the two layers of smooth muscle. The vascular smooth muscle again exhibited a high PAS-positive reaction.

The serosa was found on the outer surface of the myometrium. It consisted of a thin matted layer of connective tissue covered by a simple squamous mesothelial cell layer. The connective tissue demonstrated elastic, reticular, and

collagen fibers with venules and arterioles that caused a bulging of the mesothelial layer.

B. Extracted Controls

Extraction with cold 1 M saline caused changes in the morphology of the histological constituents and in the staining reactions. A slight fraying of the mucosal epithelium was noted. Smooth muscle cells and fibroblasts were shrunken to variable degrees. Nuclei were somewhat pycnotic and collagen fibers appeared clumped or more closely arranged. Interstitial spaces were abundant in the connective tissue layers and in the myometrium (Figure 9). Elastic fiber distribution appeared normal while the reticular nets in the myometrium appeared as hollow shells due to the cell shrinkage (Figure 10). The PAS-positive film coverning the mucosa persisted. However, the reaction in the smooth muscle cells of the vessels and the longitudinal myometrial layer appeared decreased. The shrunken smooth muscle cells of the circular layer retained the cytoplasmic PAS-positivity (Figure 11). No descriptive differences were observed when sections of first, second, and third biopsy tissue were compared.

C. Nonextracted Experimental

This tissue exhibited the typical morphological characteristics of the nonextracted control tissue. However, the normally basophilic cytoplasm of the mucosal epithelium was noted to have a slight acidophilia when observed with the hematoxylin and eosin stain.

D. Extracted Experimental

Extraction of BAPN treated tissue produced severe fraying of the luminal borders of the mucosal epithelial cells and a removal of the PAS-positive film (Figure 12). Slight acidophilia of the mucosal epithelial cytoplasm persisted. A disruption and decrease in the reticular fibers were noted. These changes were more evident in the longitudinal myometrial smooth muscle cells when compared with tissue from all BAPN treated animals (Figure 13). In the third biopsy, tissue from BAPN treated animals demonstrated concentrations of macrophages, eosinophils, and lymphocytes in the interstitial spaces around the vessels (Figure 14).

III. AUTORADIOGRAPHIC OBSERVATIONS

A. Silver Grain Distribution

Silver grain invariably were found over every portion of the tissue. A small number of silver grains was noted over the mucosal epithelial cells. The mucosal connective tissue exhibited a moderate number of silver grains distributed in a non-uniform manner (Figure 15). The myometrium of nonextracted tissue demonstrated a uniform silver grain distribution that was considerably heavier than that over other areas of the tissue (Figure 16). However, in the saline extracted control and experimental tissue, the uniformity in distribution was absent due to the occurrence of large tissue spaces caused by the saline extraction (Figure 17).

No apparent differences in the distribution of silver grains were noted when the extracted and nonextracted, experimental and control tissues were compared.

B. Results of Silver Grain Counts

Statistical analysis of data significance was made and is contained in Appendix II.

Tables 1 and 2 illustrate the percentage increase or decrease exhibited by the values obtained for extracted control and experimental tissue when compared to the values obtained from non-extracted control and experimental tissue. It can be seen that when the values obtained for the extracted first biopsy control tissue were compared to the nonextracted values, in all instances there was a percentage increase in silver grain counts per unit area. Whereas, similar comparisons of second biopsy results demonstrated a percentage loss of silver grains in three of four animals. Due to necessary sacrifice of three of the control animals prior to the third biopsy, only one value was obtained for third biopsy tissue. The single value obtained for this biopsy was 32 percent higher than the nonextracted control value.

Comparison of experimental values demonstrated that there was a percentage increase in the majority of extracted experimental tissue when compared to nonextracted experimental tissue. One value for the first biopsy was equal to the nonextracted experimental value while one value for each of the second and third biopsies showed a decrease when compared to

TABLE 1.--Percentage variation of average counts per 0.06 μ^2 for extracted control biopsies when compared to respective nonextracted biopsies.

Control Animal	First Biopsy		Second Biopsy		Third Biopsy	
	Non Extracted	Extracted	Non Extracted	Extracted	Non Extracted	Extracted
1	*	+37%	*	- 18%	*	-
2	*	+27%	*	- 15%	*	-
3	*	+43%	*	- 56&	*	+27%
4	*	+25%	*	+135%	*	-

* Nonextracted biopsy was considered as 100 percent to obtain comparisons.

Positive and negative symbols to the left of value designates respective increase or decrease from the nonextracted value.

TABLE 2.--Percentage variation of the average silver grain counts per 0.06 μ^2 for saline extracted experimental biopsies when compared to respective nonextracted biopsies.

Experimental Animal	First Biopsy Non Extracted	First Biopsy Extracted	Second Biopsy Non Extracted	Second Biopsy Extracted	Third Biopsy Non Extracted	Third Biopsy Extracted
5	*	+30%	*	+20%	*	+32%
6	*	0%	*	+32%	*	- 5%
7	*	+50%	*	+12%	*	+29%
8	*	29%	*	-10%	*	+37%

* Nonextracted biopsy was considered as 100 percent to obtain comparison

Positive and negative symbols to the left of value designates a respective increase or decrease from nonextracted value.

the nonextracted values. The decreased values of 5% and 10% were not extreme. These data indicated that saline extraction did not cause extraction of the label in BAPN-treated animals, at least as demonstrated by this technique.

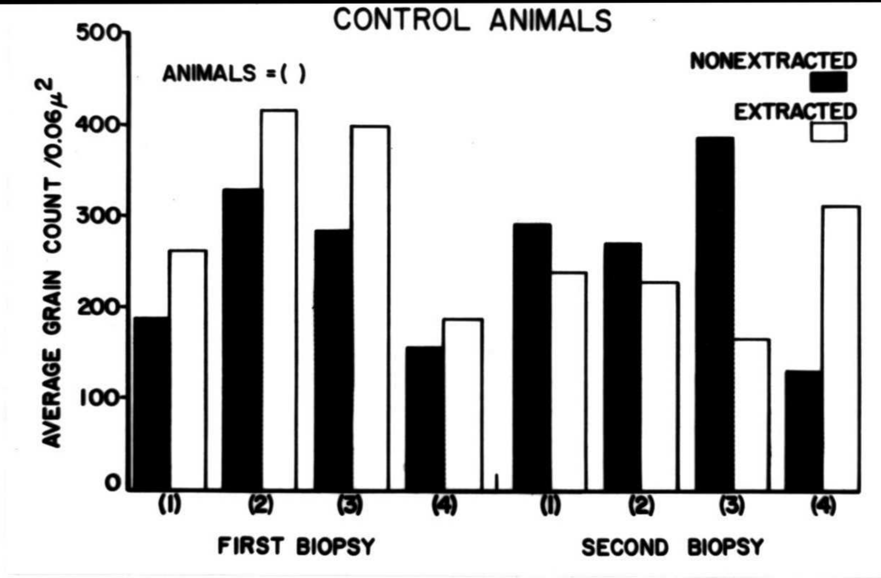
Graphs I, II and III illustrate the actual grain counts obtained for all individual biopsies. From these graphs it can be seen that there was considerable variation among counts of separate biopsies from the same animal as well as between different animals in both the BAPN treated and control groups.

When total grain counts for nonextracted and extracted control and experimental tissues from each biopsy were averaged, it was found that the experimental averages were lower than control averages in all cases but one (see Table 3). Furthermore, the averages obtained for extracted BAPN-treated and control tissue were higher than the non-extracted values in all but one case. These results are illustrated in Table 3.

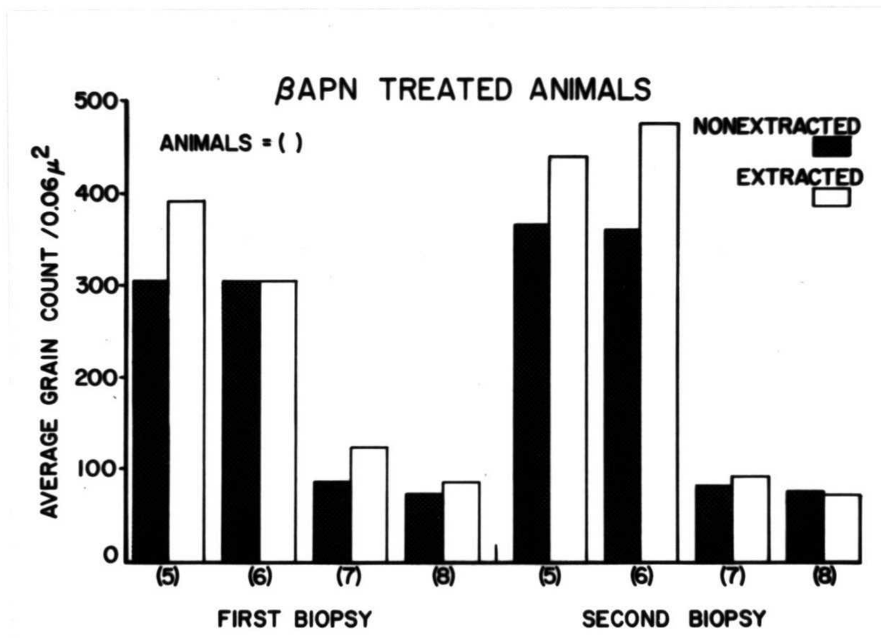
TABLE 3.--Comparison of average silver grain counts per 0.06 μ^2 for each biopsy.

Number of Biopsy	Control Nonextracted	Control Extracted	Experimental Nonextracted	Experimental Extracted
1	268	320	193	230
2	273	236	223	271
3	295*	375*	168	204

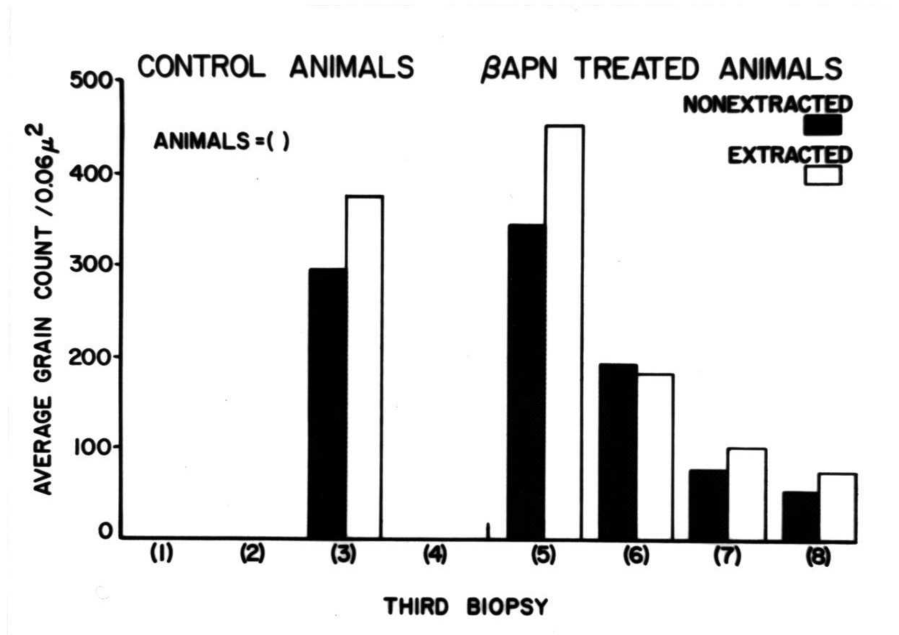
*these values are for one animal.



GRAPH II.--Bar graph of silver grain counts for the first and second biopsies of each experimental animal.



GRAPH III.--Bar graph of silver grain counts for the third biopsy of each control and experimental animal.



CHAPTER IV

DISCUSSION

It has been proposed that beta-aminopropionitrile and other lathyrogenic agents affect the inter- and intra-molecular linkages of the collagen either by altering key chemical bonding groups of newly synthesized collagen or by cleaving or weakening these bonds in the mature preformed collagen fiber (21). Newly synthesized collagen has generally been accepted as the primary source of the increased saline-soluble collagen fraction noted in lathyric rats. However, evidence has been presented indicating an effect of lathyrogenic agents on collagen in all stages of maturation but to varying degrees (21). Page and Benditt (42) suggested that the preformed collagen fiber did not contribute to the saline-soluble collagen from whole minced lathyric chick embryos. Smiley et al. (34) came to the same conclusions. However, Tanzer and Gross (29), using the long bones of lathyric chick embryos, produced data showing contribution to the saline soluble collagen fraction from both newly synthesized and preformed collagen. Levene and Gross (32), using skin, bone, and aorta of chick embryos, again found a contribution from the preformed fibers. Consequently, there is still some question concerning the effect of BAPN

or any other lathyrogenic agent upon the mature collagen fiber. The possibility exists that this variability is the result of experimental studies on collagen of different turnover rates and therefore reflects the degree of anabolic activity in the connective tissue rather than the actual state of the collagen fiber.

It is well established that the ground substance plays an important role in the maturation and stabilization of the collagen fiber (34). It also plays an important role in the catabolic activity involved in the "casting-off" of unwanted collagen (4). Therefore, it seems reasonable to assume that connective tissue with a high turnover of collagen would contain a ground substance somewhat different from that of connective tissue with a slow collagen turnover. The role of ground substance in the metabolism of its fibrous constituents is still unclear, but it is thought that in some way the fiber precursor is bound in the ground substance during its orientation and bonding (4). The possibility exists that the nature of the ground substance controls the rate at which collagen matures and gains stability. Therefore, another site of possible lathyrogenic action could be in that element of the ground substance controlling maturation and stabilization which would be present to a greater degree in the connective tissue showing high collagen turnover. The uterus of the pregnant rat was chosen for this study because it possesses collagen of the highest turnover rate of all tissues thus far studied.

Young female albino rats weighing an average of 200 grams were chosen for this investigation because it was reported that young animals are more susceptible to lathyrogens (27). It was felt that the effect of the lathyrogenic agent would be more severe and the effect easier to detect and measure in young rats. The animals had not been previously mated to avoid residual buildup of the uterus from previous gestation (18).

A dosage of L-proline-H^3 of 0.5 μc/gram body weight was found sufficient to label the uterine collagen. The time of 36 hours which was allowed for the turnover of collagen from the soluble to its insoluble state was considered sufficient due to information previously recorded on collagen turnover rates in rat uterine tissue (18). If any significant amount of L-proline-H^3 labeled collagen had remained in its soluble state, it would have been washed out of the tissue with saline extraction. Since this did not occur, the collagen studied in this experiment had at least matured past the saline-soluble stage.

The seventeenth to the nineteenth days of gestation were selected for experimentation because the most pronounced collagen increase in uterine tissue was reported during the latter days of gestation (18). It has been reported that BAPN does not produce uterine involution and fetal resorption in the prepartum rat before the seventeenth day of gestation (24). These characteristics were reported to be the extreme reaction of the pregnant rat to the lathyrogenic

agent. An extreme effect such as this was undesirable in this study but was thought to be avoided by harvesting of tissue within the shortest reported time of BAPN action.

The extreme variation in range noted in silver grain counts of tissue from the different animals was thought to be caused in part by individual variation and in part by the method of gestation timing. Daily vaginal lavages introduced a possible timing error of up to 24 hours. This error was believed to have been particularly important in the autoradiographic measurements of mature collagen during the final days of gestation since at that time fetal growth was greatest.

The effect of estrogen in inhibiting the action of the lathyrogenic agent was not considered to be an influential factor in this study because of the high dosage of BAPN given the animals. The dose given the pregnant rat was 1 milligram per gram body weight with the average total dose of 300 milligrams. In the study by Simmons et al.(25) involving estrogen inhibition by aminoacitonitrile (AAN), the total dose given to mice over a period of six days was 120 milligrams. Dosages used in other studies (30) involving BAPN were smaller, often not reaching half the amount given in the present study. The acidophilia of the mucosal epithelial cytoplasm and the apparent increased disruption of the reticular nets and fibers demonstrated histological evidence of an effect of BAPN.

The presence of fluctuation in the counts of different biopsies for the same animal was thought to be caused by the

fixation of the uterine tissue during different stages of uterine contraction. Motility of the uterine tissue has been reported to occur to varying degrees at all times during the estrus and prepartum stages of the uterus (44). Presuming fixation produced a preserved tissue similar to the live state, the contracted state of the uterus would yield higher silver grain counts due to the more compact tissue. Likewise, in the flaccid state, the tissue would yield lower counts as there was less compact tissue. The data suggest a greater degree of variation in the silver grain counts for control animals compared with experimental animals thus suggesting greater uterine motility. The degree of motility of uterine tissue is believed to be intimately associated with the estrogen activity (44). The greater motility suggested in the uterine tissue of control animals compared to the experimental animals leads the author to conclude that BAPN acts to inhibit or neutralize the effect of the estrogen. These conjectures demand further study and more conclusive data and, therefore, should be interpreted with caution.

When comparing average silver grain counts of saline and nonsaline extracted tissue for both experimental and control animals, a lower grain count was recorded in experimental animals. The possibility exists that BAPN caused solublization of the collagen during the two hours before the first biopsy was taken and thus accounted for lower average counts for the experimental group. Increases in hydroxyproline content of blood and urine have been reported shortly

after BAPN administration (45). However, the present research utilized no methods which could confirm those findings.

The data of this study demonstrated that extraction with cold 1 M saline did not remove the L-proline-H^3 label from either the control or BAPN treated tissue. Moreover, the consistent increase of silver grains in both control and experimental saline-extracted tissue indicated that some factor not influenced by the experimental conditions was exerting an effect upon the data. Such an effect could be explained by the shrinkage of the tissue components observed by light microscopy. The 1 M saline used for extraction was hypertonic to the tissue and, therefore, was believed to have caused the shrinkage. Consequently, high silver grain counts for most saline extracted biopsies were recorded.

According to the previous literature, an alteration or cleavage of mature insoluble collagen should have been demonstrated by lower silver grain counts in the saline extracted BAPN-treated tissue. The values for the nonextracted BAPN-treated tissue and for the extracted and nonextracted control tissue should have been comparable because each should have possessed a relatively equal quantity of saline-unaffected labeled collagen. The data show when comparing these four groups, that there was no significant detectable decrease by the saline extraction of BAPN-treated tissue utilizing the techniques of this study. Therefore, the study suggests that there is no effect upon the mature saline insoluble collagen of the prepartum rat uterus.

The above findings do not invalidate work of other authors. However, it should suggest the use of a too liberal interpretation of the term "mature-preformed" or "insoluble" collagen be avoided. The overlap of tropocollagen aggregates present in the different extractable portions of collagen shows a heterogenity of these fractions. Failure to consider this heterogenous nature and also the variable turnover rate of the collagen is believed to have led to the conflicting data. The creation and use of more meticulous definitions for the various states and extractable fractions of collagen are suggested for further study involving collagen metabolism on the molecular level.

CHAPTER V

SUMMARY AND CONCLUSIONS

Ten female albino rats of the Sprague-Dawley strain were used in a study to determine the effect of beta-aminopropionitrile on the mature collagen of the prepartum rat uterus. The rats were mated at sexual maturity with the day of sperm discovery in vaginal lavages recorded as day zero of pregnancy. On the seventeenth day of gestation each animal was given an intraperitoneal injection of L-proline-H^3. Thirty-six hours after L-proline-H^3 injection, half of the rats were given intraperitoneal injections of beta-aminopropionitrile and the other half were given injections of equal quantities of physiological saline. Two hours post-BAPN injection, laparotomy was performed and biopsies consisting of the entire distal implantation site (fetus, placenta, and uterus) were taken from each uterine horn. The incision was clipped shut and the animal was allowed to revive. Surgery was repeated every hour up to and including four hours post-BAPN injection. The placenta and fetus were removed. The uterine tissue of the left horn was fixed immediately in neutral buffered 10% formalin while the tissue of the right horn was extracted with 1 M saline at 4° C for 24 hours prior to fixation. Paraffin embedded tissue was

cut at 6 microns and mounted on chemically cleaned glass slides for autoradiography. Other representative uterine tissues were prepared for selected histological stains.

Histological descriptions of nonsaline extracted experimental and control tissue for all biopsies were found to differ only with regard to staining reactions. A PAS positive reaction in the cytoplasm of the myometrial smooth muscle cells was found extremely variable in all biopsies of both experimental and control animals. Increased acidophilia of the normally basophilic cytoplasm of mucosal epithelial cells was observed for BAPN treated tissue.

Saline extraction of experimental and control tissue caused severe shrinkage of smooth muscle cells in the myometrium. Fibroblasts appeared shrunken and nuclei appeared pycnotic. Collagen fibers were more closely arranged. Fraying of the luminal surfaces of the mucosal epithelial cells was apparently more severe in BAPN treated tissue. A severe disruption and apparent removal of reticular fibers and nets were observed in the myometrium of BAPN treated tissue.

Silver grain distribution appeared uniform in the myometrial layers of nonsaline extracted tissue, but was nonuniform in the mucosa, serosa, and in the connective tissue layer between myometrial strata. Saline extraction produced abundant tissue spaces which interrupted this uniformity. Silver grain counts for control and experimental tissue for the first biopsy showed a higher count for saline

extracted tissue. Correlating this with the microscopic observations suggested that the hypertonic 1 M saline solution used for extraction caused a persistant tissue shrinkage.

The lack of exact gestation timing was believed to be the reason for extreme variations in ranges of silver grain counts for all animals. However, fluctuations in the form of increase or decrease in silver grain counts when comparing sequential biopsies for the individual animals were believed to be due to the fixation of the tissue during different contractile states. The fluctuations were found to be more divergent in the control tissue. A suggestion of an inhibitory effect of BAPN on the ability of estrogen to produce motility could not be confirmed with the data.

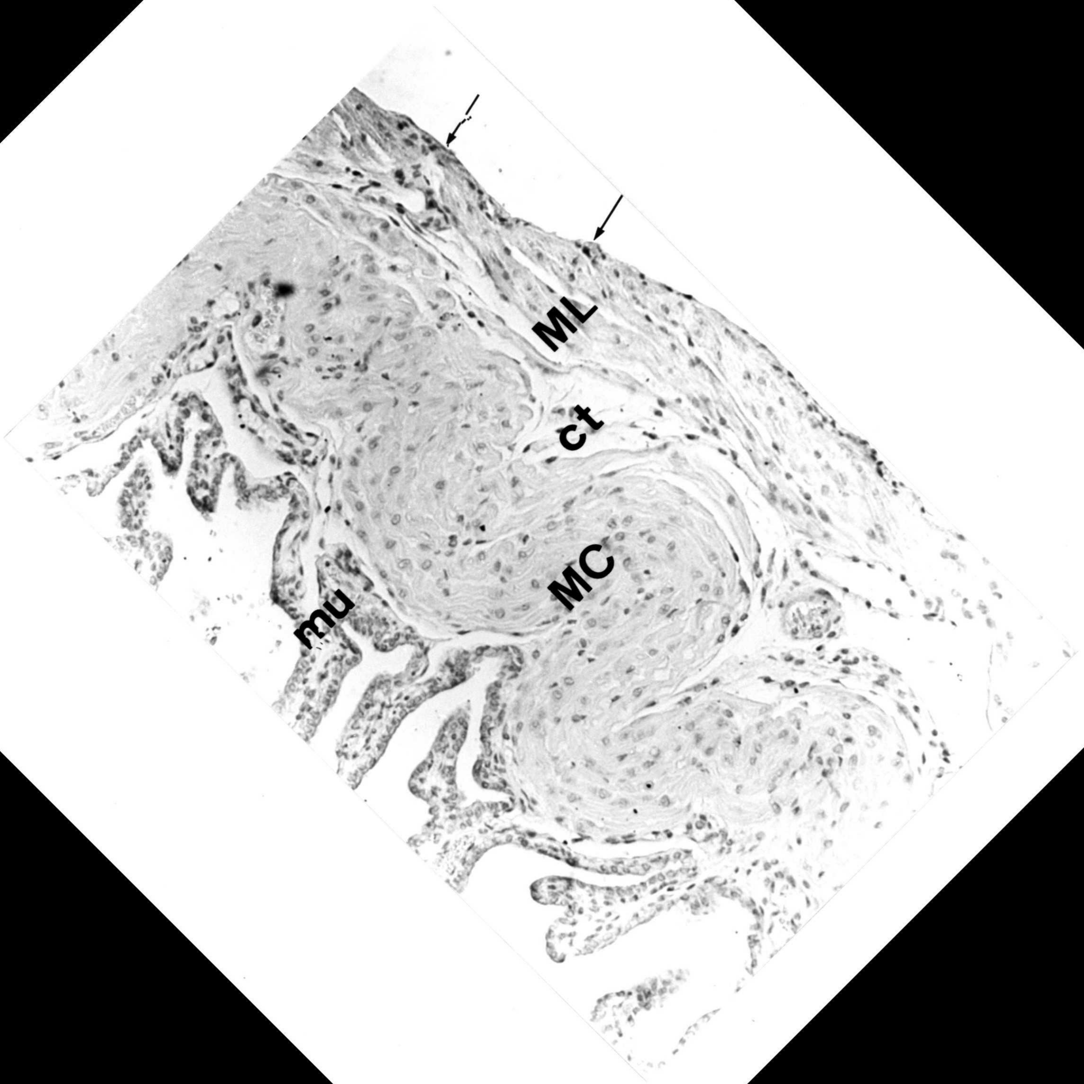
To determine the presence of an action of BAPN on the labeled uterine collagen, comparisons were made of average silver grain counts from extracted and nonextracted tissue of the experimental and control animals. Because there was no change in silver grain count for nonextracted control tissue, it was assumed that all of the label present in both the extracted and nonextracted control tissue was incorporated in the saline insoluble collagen.

A lower silver grain count average was noted for experimental tissue. Therefore, the possibility of BAPN acting on uterine collagen before the first biopsy was considered. However, if the BAPN altered or cleaved the bonds of the mature collagen, a lower silver grain count for

extracted BAPN treated tissue should have been evident when compared to the other tissue. In this study a lower silver grain count was not recorded for saline extracted BAPN treated tissue. Therefore, the data suggest that BAPN does not cause an alteration or cleavage of bonds of the saline insoluble collagen of the prepartum rat uterus.

PLATE I

Figure 1. Photomicrograph of longitudinal section of normal rat uterus adjacent to implantation site, mu., mucosa or endometrium; MC., circular layer of myometrium; ct., connective tissue between myometrial layers; ML., longitudinal layer of myometrium; serosa designated by arrows. Hematoxylin and eosin. X.800



ML
ct
MC
mu

PLATE II

Figure 2. Photomicrograph of mucosa from non-extracted control tissue showing epithelium and adjacent connective tissue layer. Note the basally located nuclei, the syncitial appearance of epithelial cell boundaries, and variation of epithelial types. Ep., epithelium of mucosa; Ct., subepithelial connective tissue; MC., circular layer of myometrium. Masson's trichrome stain. X.1000

Figure 3. Photomicrograph of mucosa of nonextracted control tissue showing PAS positive film on the luminal surface of epithelium, and fibrillar basement membrane. Ep., epithelium; Ct., connective tissue; single arrows designate PAS positive film; double arrows designate basement membrane. Periodic acid-Schiff. X.1000

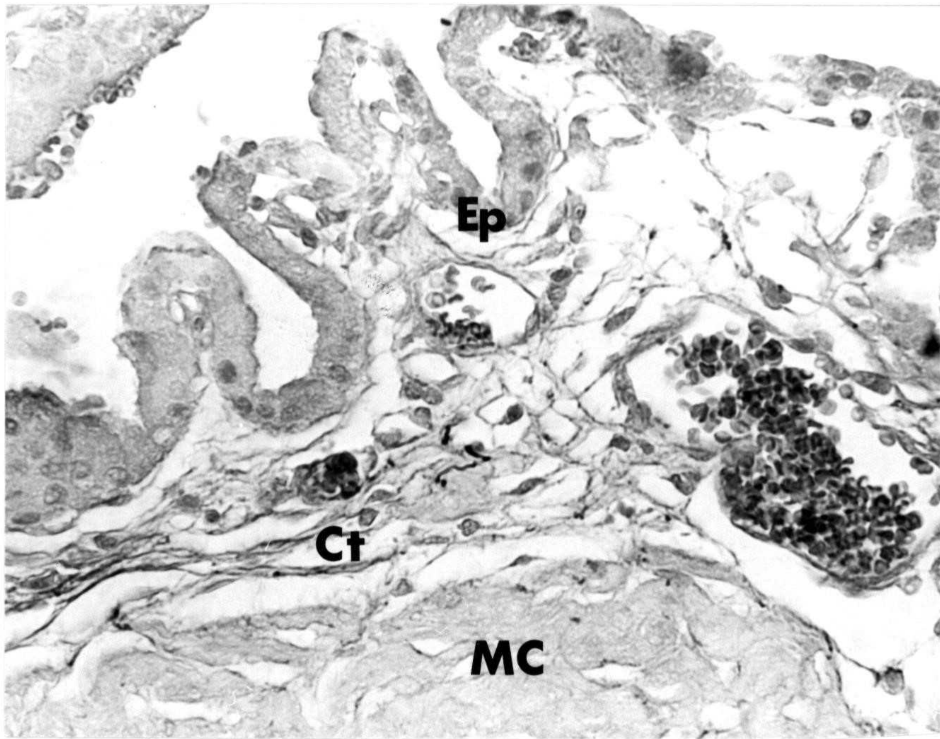


Figure 2

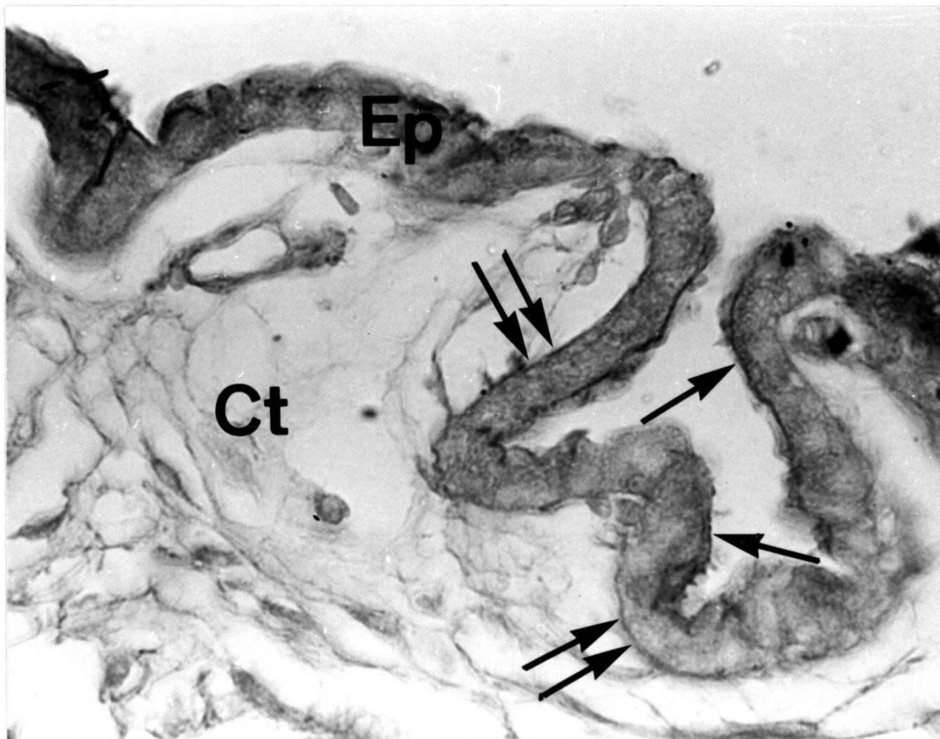


Figure 3

PLATE II

PLATE III

Figure 4. Photomicrograph of mucosa of nonextracted control tissue showing concentration of reticular fibers near the base of the epithelial cells. Ep., epithelium; Ct., subepithelial connective tissue. Arrows designate concentration of reticular fibers. Snook's reticulin stain. X.1600

Figure 5. Photomicrograph of mucosa of nonextracted control tissue showing cells and vessels in the subepithelial connective tissue. Note the vessel immediately subjacent to the epithelium. Ep., epithelium of mucosa; Ct., subepithelial connective tissue; BV., blood vessel; MC., circular myometrial layer. Hematoxylin and eosin. X.1600

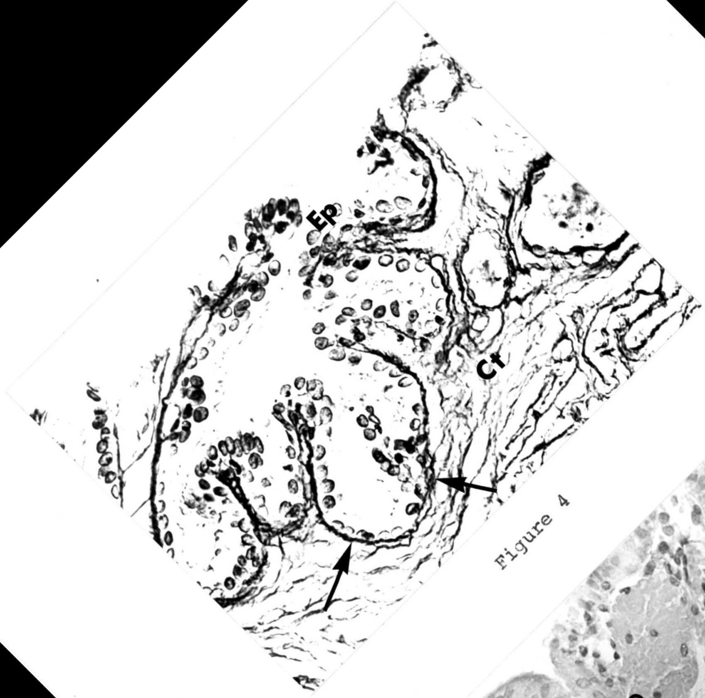


Figure 4

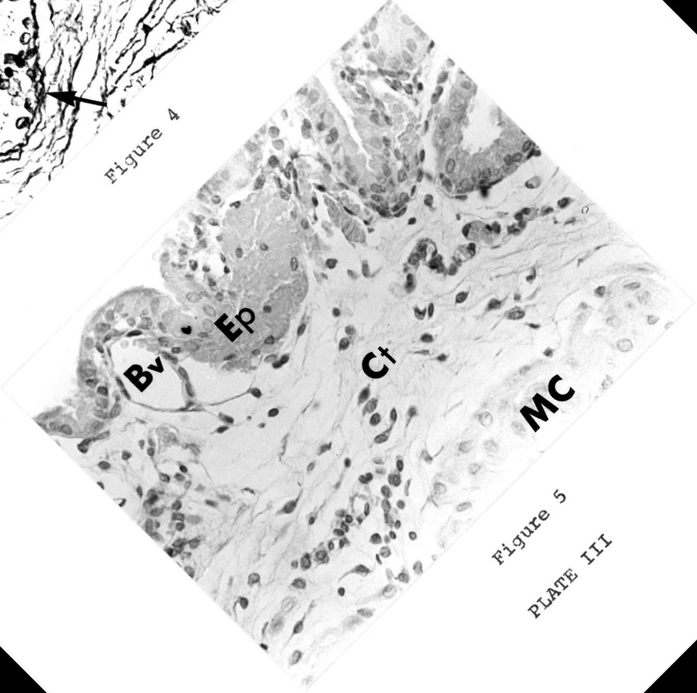


Figure 5

PLATE III

PLATE IV

Figure 6. Photomicrograph of a vessel from a nonextracted control animal showing the PAS positivity in the vascular smooth muscle. Note the reaction present in the adjacent myometrium. Bv., Blood vessel; MC., circular layer of myometrium. Periodic acid-Schiff. X.1600

Figure 7. Photomicrograph of nonextracted control tissue showing the reticular fibers and nets in the longitudinal layer of myometrium. Connective tissue between smooth muscle layers contains cut portions of isolated smooth muscle cells. MC., Circular layer of myometrium. ct., connective tissue between layers of the myometrium. ML., longitudinal layer of myometrium. Sm., Isolated smooth muscle cells. Snook's reticulin stain. X.1600

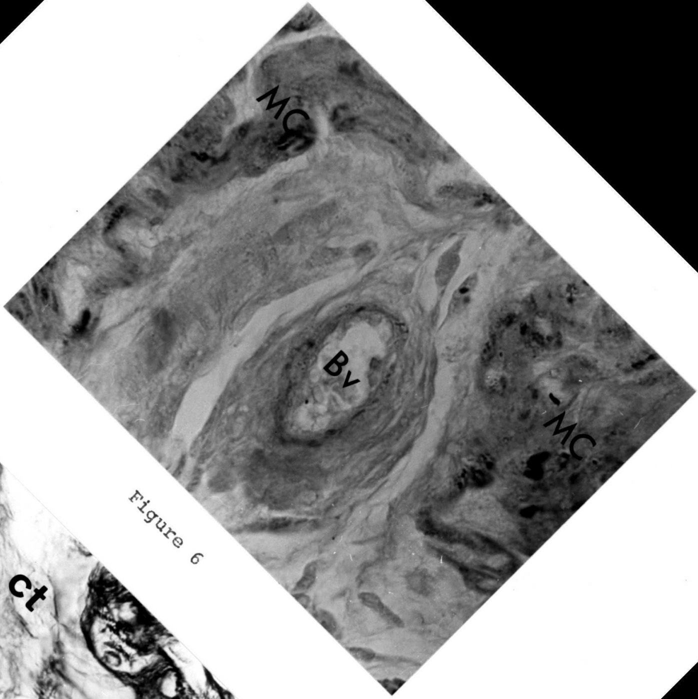


Figure 6

Figure 7

PLATE IV

PLATE V

Figure 8. Photomicrograph of longitudinal section of nonextracted control tissue showing PAS reaction in the circular myometrium. Note the more intense reaction in the circular myometrial layer. mu., mucosa; MC., circular layer of myometrium; ct., connective tissue between myometrial layers; ML., longitudinal layer of myometrium. Periodic acid-Schiff. X.800

Figure 9. Photomicrograph of mucosa and circular layer of myometrium of control tissue showing the effects of saline extraction. Note shrunken smooth muscle cells and fibroblasts with pycnotic nuclei. Also note abundant tissue spaces in mucosal connective tissue and in myometrium. Ep., mucosal epithelium; Ct., subepithelial connective tissue; MC., circular myometrial layer. Masson's trichrome. X.1000

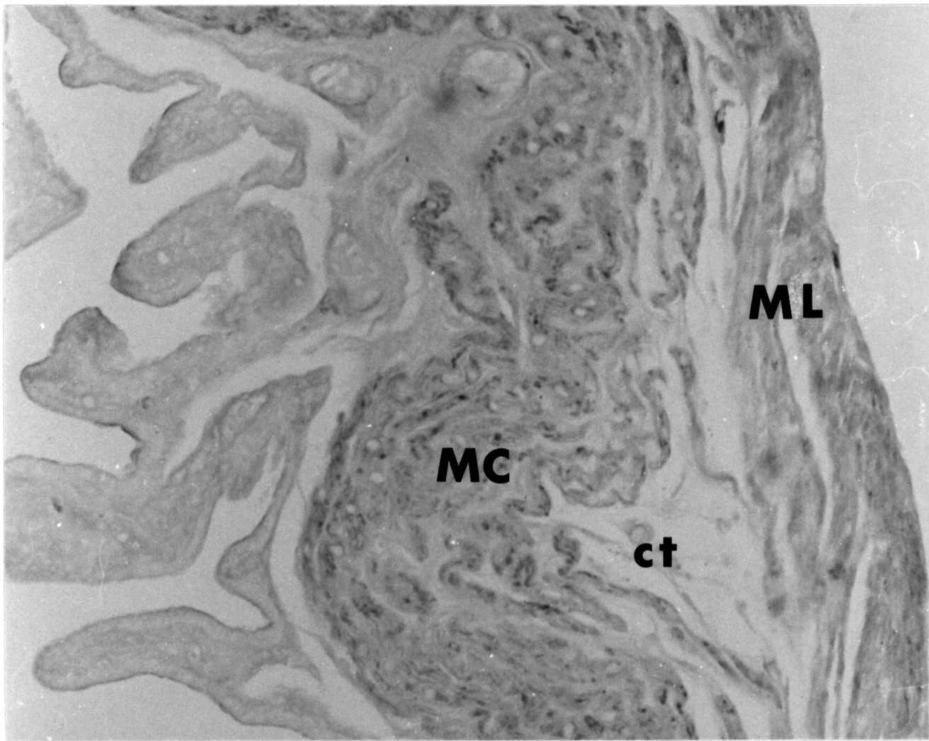


Figure 8

Figure 9

PLATE V

PLATE VI

Figure 10. Photomicrograph of myometrium of saline extracted control tissue showing reticular arrangement after saline extraction. Note the hollow shell appearance of the reticular nets. MC., circular myometrial layer; ML., longitudinal myometrial layer. Snook's reticulin stain. X.1600

Figure 11. Photomicrograph of the myometrium of saline extracted control tissue showing effect of extraction on the PAS reaction in the smooth muscle. Note excess tissue spaces in the myometrium. Also note retention of PAS reaction by the cells of circular smooth muscle layer and loss of reaction in the longitudinal smooth muscle layer. MC., circular myometrial layer; ML., longitudinal myometrial layer. Periodic acid-Schiff. X.1000

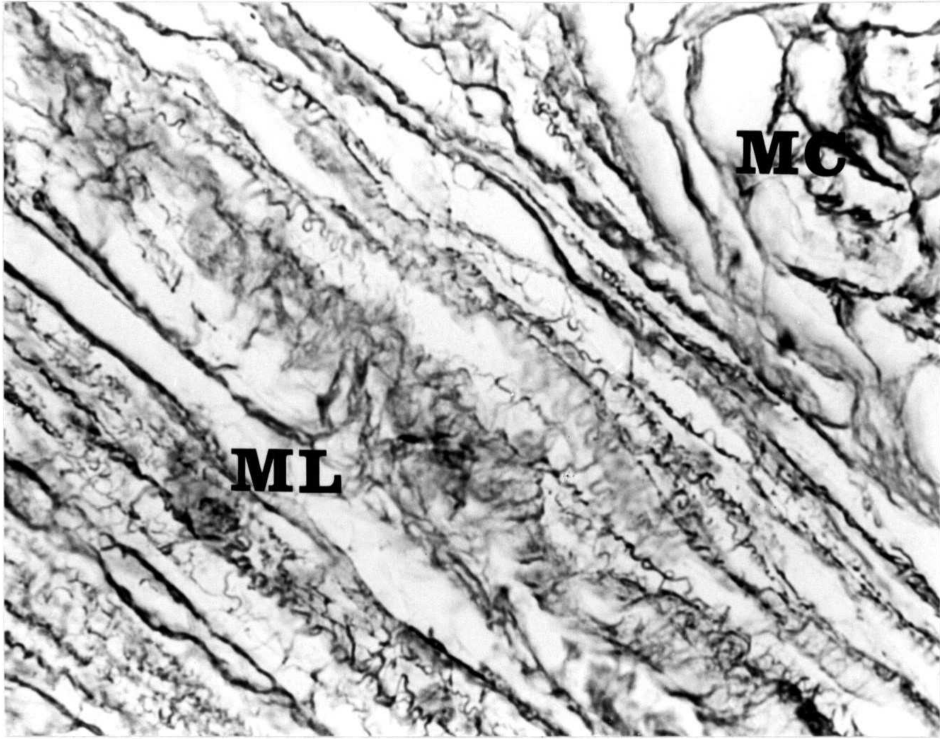


Figure 10

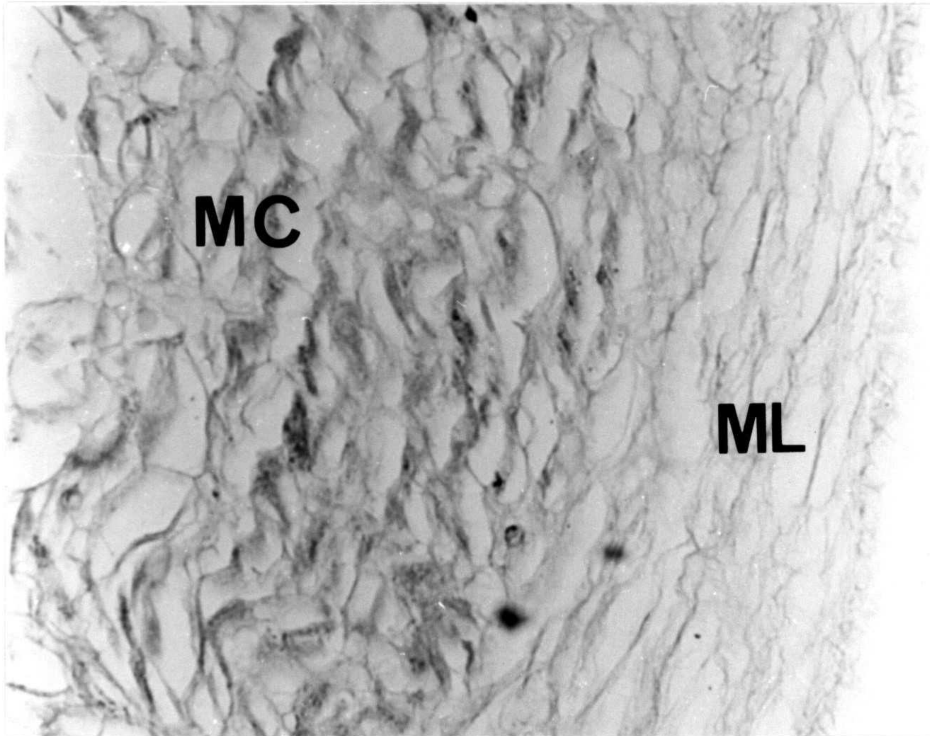


Figure 11

PLATE VI

PLATE VII

Figure 12. Photomicrograph of mucosa from extracted BAPN treated tissue showing fraying of epithelium and removal of PAS positive film. Periodic acid-Schiff. X.1000

Figure 13. Photomicrograph of saline extracted BAPN treated tissue showing a disruption of the reticular fiber and net arrangement in the longitudinal layer of myometrium. Compare this figure with Figure 10. MC., circular myometrial layer. ML., longitudinal myometrial layer. Snook's reticulin stain. X.1000

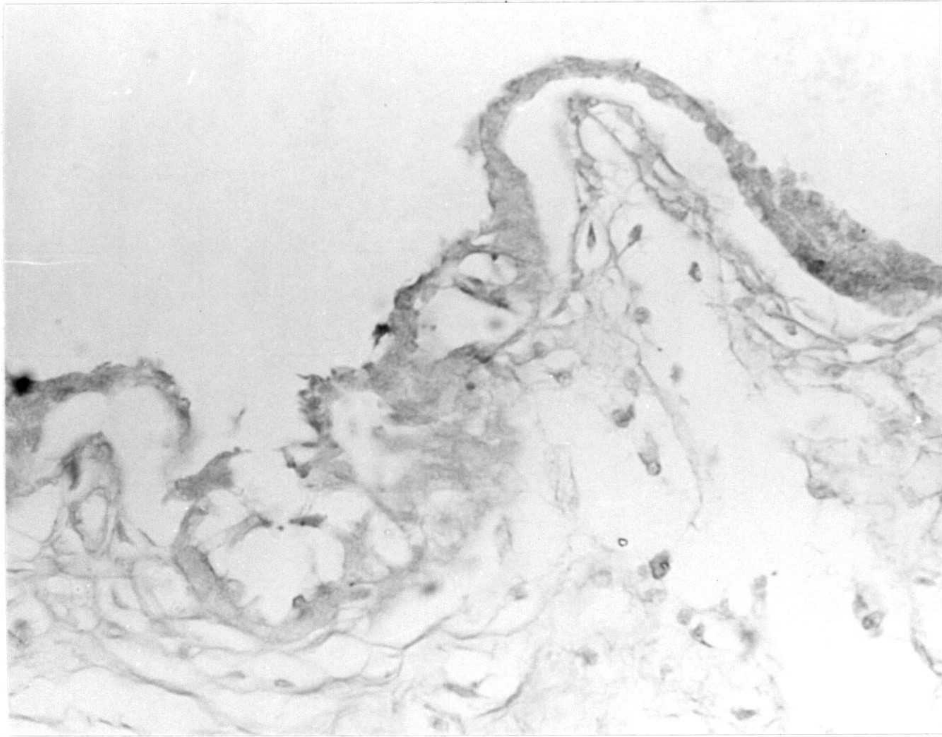


Figure 12

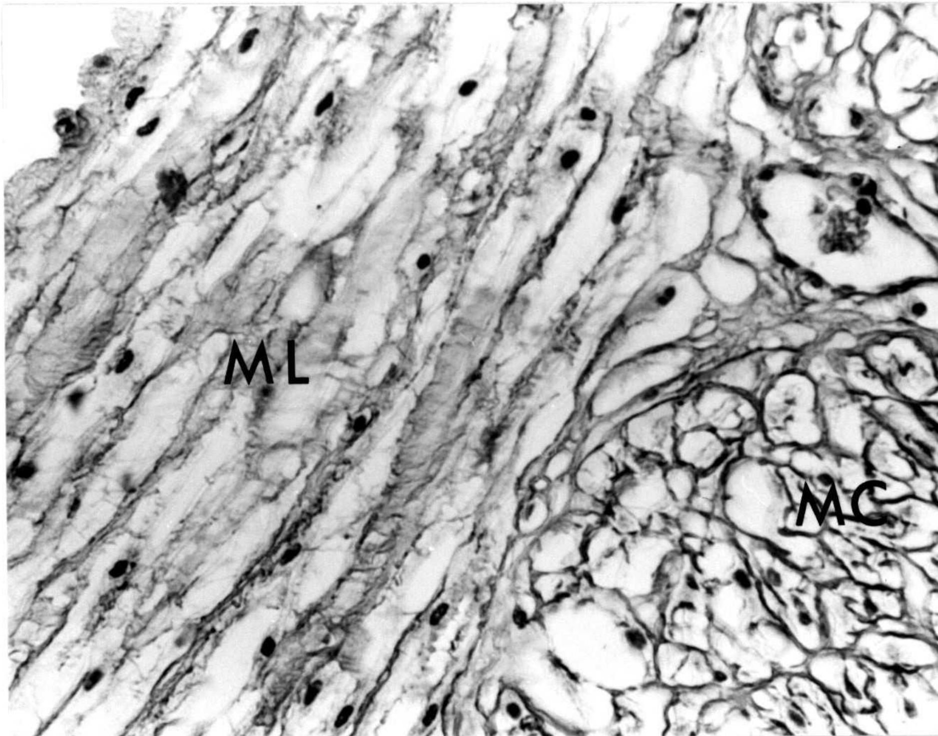


Figure 13

PLATE VII

PLATE VIII

Figure 14. Photomicrograph of section of uterine tissue from the third biopsy of BAPN treated animal showing blood vessel (Bv) and the concentration of leucocytes in the interstitial spaces. Hematoxylin and eosin. X.1600

Figure 15. Photomicrograph of an autoradiograph of tissue from a nonextracted control animal showing silver grain distribution of mucosa. Ep., mucosal epithelium; Ct., mucosal connective tissue. Periodic acid-Schiff-Hematoxylin. X.1600

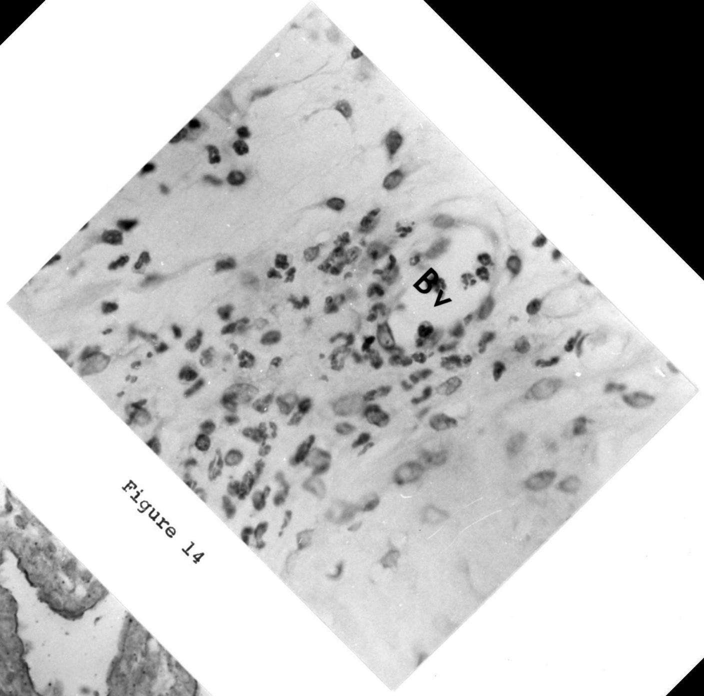


Figure 14

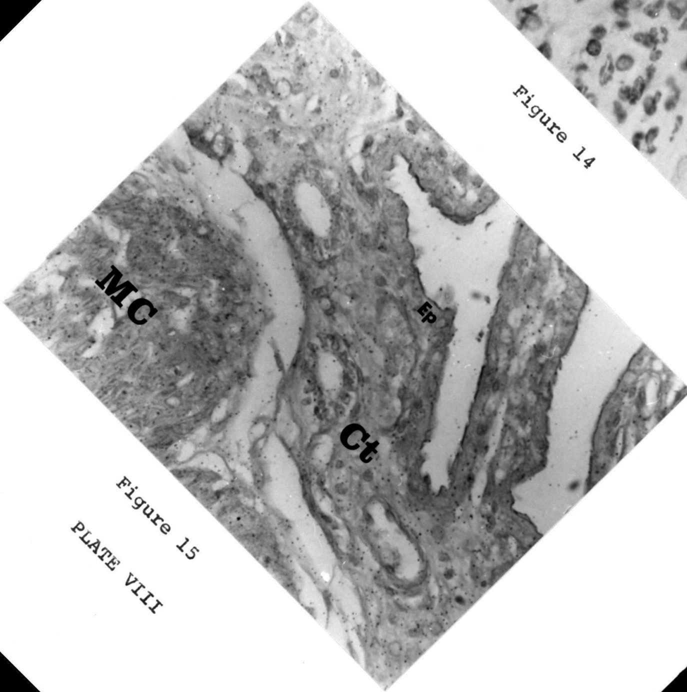


Figure 15

PLATE VIII

PLATE IX

Figure 16. Photomicrograph of an autoradiograph of tissue from nonextracted control animal showing silver grain distribution of circular layer of myometrium. BV., blood vessels; MC., circular layer of myometrium. PAS-hematoxylin. X.1600

Figure 17. Photomicrograph of an autoradiograph of saline extracted tissue from a BAPN treated animal showing increased tissue spaces and cell shrinkage in the longitudinal layer of the myometrium. S, serosa; ML., longitudinal layer of myometrium. PAS-hematoxylin. X.1600

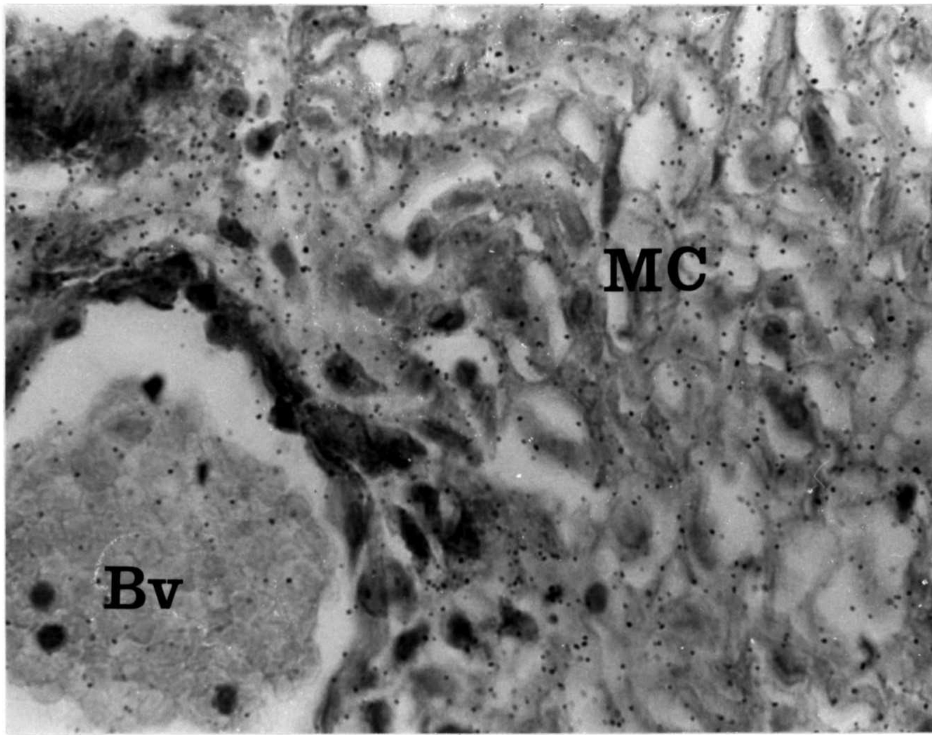


Figure 16

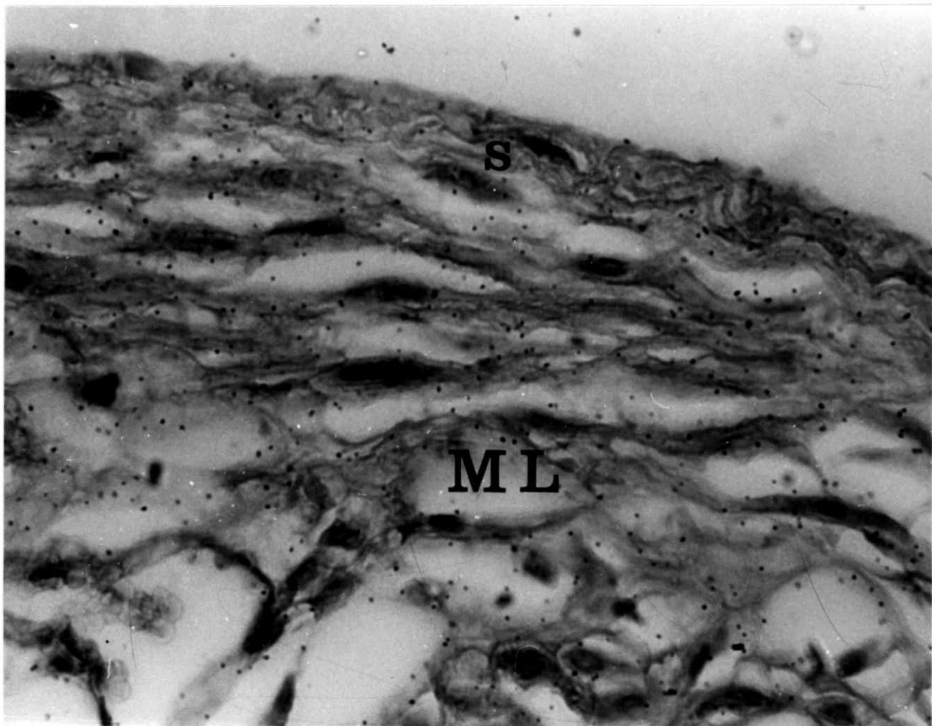


Figure 17

PLATE IX

APPENDIX II

TABLE 4.--"T" test of significance of nonextracted and extracted experimental average silver grain counts per biopsy.

Animal Number	Nonextracted Average grain count per biopsy	Nonextracted Deviation from mean (d)	Extracted Average grain count per biopsy	Extracted Deviation from mean (d)
5	305	113	395	161
	368	176	440	106
	345	153	455	221
6	305	113	305	71
	360	168	475	241
	191	2	185	49
7	85	108	125	109
	85	108	95	139
	80	113	100	134
8	75	118	85	149
	55	138	75	159
	55	138	75	159

Total/N=μ Total/N=$\bar{X}$

μ=193 $s=\sqrt{\Sigma d^2/N}$ x=234 $s=\sqrt{\Sigma d^2/N}$

s=128 s=151

Mean value for nonextracted experimental is 193 with standard deviation of $\pm$ 128.

Mean value for extracted experimental is 234 with standard deviation of $\pm$ 151.

$$T = \frac{\bar{X} - \mu}{s/\sqrt{N}}$$

T = .9676

P =>0.2 Not Significant

LITERATURE CITED

1. Bloom, W., and Fawcett, D. W. A Textbook of Histology. W. B. Saunders Co., Philadelphia. 1964.

2. Rhodin, J. A. G. An Atlas of Ultrastructure. W. B. Saunders Co., Philadelphia. 1963.

3. Gersh, I., and Catchpole, H. R. The Nature of Ground Substance of Connective Tissue. Perspec. In Biol. and Med. 3:284-301. 1960.

4. Houck, J. C. Connective Tissues and the Collagen Diseases. Clinical Proc. 17:243-252. 1961.

5. Taylor, J. J., and Yeager, V. L. The Structure of Elastic Fibers in the Fibrous Periosteum of the Rat Femur. Anat. Rec. 156:129-142. 1966.

6. Snook, T. The Guinea Pig Spleen. Studies on the Structure and Connections of the Venous Sinuses. Anat. Rec. 89:413-427. 1944.

7. Piez, K. A., and Gross, J. The Amino Acid Composition and Morphology of Some Invertebrate and Vertebrate Collagens, Biochem. Biophys. Acta 34:24-39. 1959.

8. Gross, J. Collagen. Sci. Amer. 204:121-130. 1961.

9. Cooper, G. W., and Prockop, D. J. Accummulation of Protocollagen and Extrusion of Collagen by Embryonic Chondrocytes. (Abstr.) Anat. Rec. 157:231. 1967.

10. Harrington, W. F., and McBride, O. W. The Stabilization of Collagen, Structural Organization of the Skeleton. Birth Defects Original Article Series II: 1966.

11. Piez, K. A. Crosslinking of Collagen, Structural Organization of the Skelton. Birth Defects Original Article Series II: 1966.

12. Keech, M. K. The Formation of Fibrils from Collagen Solution. J. Biophys. and Biochem. Cytol. 9:193-203. 1961.

13. Bornstein, P. Crosslinking of Collagen Chains. Fed. Proc. 25: 1966.

14. Veis, A., and Anesey, J. Modes of Intermolecular Cross-linking in Mature Insoluble Collagen. J. Biol. Chem. 240:3899-3910. 1965.

15. Bornstein, P., and Piez, K. A. A Biochemical Study of Human Skin Collagen and the Relation Between Intra- and Intermolecular Crosslinking. J. Clin. Invest. 43: 1964.

16. White, A., Handler, P., and Smith, E. L. Principles of Biochemistry. McGraw-Hill Co., New York. 1964.

17. Kao, K., Hilkens, D. M., and McGavack, T. H. Synthesis and Turnover of Proteins in Tissue of Rats. Proc. Soc. Exp. Biol. and Med. 106:121-129. 1961.

18. Harkness, M. L. R., and Harkness, R. D. The Collagen Content of the Reproductive Tract of the Rat During Pregnancy and Lactation. J. Physiol. 123:492-500.

19. Geiger, B. J., Steenbock, H., and Parsons, H. T. Lathyrism in the Rat. J. of Nutrition 6:427-442. 1933.

20. Tanzer, M. L. Experimental Lathyrism. Internat. Rev. Conn. Tissue Res. 3:91-112. 1965.

21. Ryback, R. Inhibited Maturation of Collagen in Lathyrism as a Model for the Marfan Syndrome and its Implications. Gerontologia 11:120-126. 1965.

22. Selye, H. Influence of Various Hormones and Vitamin-D Preparations Upon Established Bone Lathyrism. Anat. Rec. 33:146-156. 1958.

23. Selye, H. Lathyrism. Rev. Can. Biol. 16:1-40. 1957.

24. Stamler, F. W. Reproduction in Rats Fed Lathyrus Peas or Aminoacetonitrile. Proc. Soc. Exp. Biol. and Med. 90:294-298. 1955.

25. Simmons, D. J., Pankouich, A. M., and Budy, A. M. Osteolathyrism in Mice and Inhibition of the Endosteal Bone Reaction in Estrogen Treated Mice by Aminoacetonitrile. Am. J. Anat. 116:367;400. 1965.

26. Wirtschafter, Z. T., and Bentley, J. P. The Extractable Collagen of Lathyritic Rats with Relation to Age. Lab. Invest. 11:365-367. 1962.

27. Van den Hooff, A., Levene, C. I., and Gross, J. Morphological Evidence for Collagen Changes in the Chick Embryos Treated with BAPN. J. Exp. Med. 110:1017-1022. 1959.

28. Tanzer, M. L., and Gross, J. Collagen Metabolism in The Normal and Lathyritic Chick. J. Exp. Med. 119:257-289. 1963.

29. Gross, J. An Intermolecular Defect of Collagen in Experimental Lathyrism. Biochem. et Biophys. Acta 71:250-255. 1963.

30. Martin, G. R., Gross, J., Piez, K. A. and Lewis, M. S. On the Intramolecular Crosslinking of Collagen in Lathyritic Rats. Biochem. et Biophys. Acta 53:599-601. 1961.

31. Levene, C. I., and Gross, J. Alterations in State of Molecular Aggregation of Collagen Induced in Chick Embryos by BAPN. J. Exp. Med. 110:771-790. 1959.

32. Follis, R. H., and Tousimis, A. J. Experimental Lathyrism in the Rat. Proc. Soc. Exp. Biol. and Med. 98:843-848. 1959.

33. Smiley, J. D., Yeager, H. and Ziff, M. Collagen Metabolism in Osteolathyrism in Chick Embryos: Site of Action of BAPN. J. Exp. Med. 116:45-48. 1962.

34. Smith, D. J., and Shuster, R. C. Collagen Biosynthesis in Norm 1 and Lathyritic Chick Embryos. Arch. Biochem. and Biophys. 98:498-503. 1962.

35. Rojkind, M., Blumenfeld, O. O., and Gallop, P. M. Isolation of an Aldehyde-containing Peptide from Tropocollagen. Biochem. Biophys. Res. Comm. 17:320-325. 1964.

36. Rojkind, M., Blumenfeld, O. O., and Gallop, P. M. Localization and Partial Characterization of an Aldehydic Component in Tropocollagen. J. Biol. Chem. 251:1530-1536. 1966.

37. Bornstein, P., Kang, A. H., and Piez, K. A. The Nature and Location of Intramolecular Crosslinks in Collagen. Proc. Nat. Acad. Sci. 55:417-421. 1966.

38. Tanzer, M. L., Monroe, D., and Gross, J. Inhibition of Collagen Intermolecular Crosslinking by Thiosemicarbazide. J. Biochem. 5:1919-1925. 1966.

39. Bensusan, H. B., McKnight, S. D., and Naidu, M. S. R. The Demonstration of a Possible Common Mechanism of Lathyrogenic Activity. Biochem. Biophys. Res. Comm. 23:128-132. 1966.

40. Rojkind M., and Juarez, H. The Nature of the Collagen Defect in Lathyrism. Biochem. Biophys. Res. Comm. 25: 1966.

41. Page, R. C., and Benditt, P. The Source of Lathyritic Collagen. Lab. Invest. 15:1643-1651. 1966.

42. Hagen, E. O., and Hagen, J. M. A Method of Inhalation Anesthesia for Laboratory Mice. Lab Animal Care 14:13-15. 1964.

43. Ponsetti, I. V. and Baird, W. A. Scoliosis and Dissecting Aneurysms of the Aorta of Rats Fed with *Lathyrus odoratus* seeds. Am. J. Path. 28:1059-1077.

44. Bo, W. J., and Atkinson, W. B. Histochemical Studies on Glycogen Deposition in the Uterus of the Rat. Anat. Rec. 113:91-96. 1952.

45. Decker, L., Levene, C. I., and Gross, J. Effect of Beta-aminopropionitrile on Free Hydroxyproline, Proline and Glycine in Chick Embryos. Proc. Soc. Exp. Biol. Med. 101:472-477. 1959.

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