1968

# A Histochemical Study of the Effects of Parathyroid Extract on Cartilage and Bone in Tissue Sections from Fetal Rat Heads

Nicholas Joseph Malinski
*Loyola University Chicago*

# A HISTOCHEMICAL STUDY OF THE EFFECTS OF PARATHYROID EXTRACT ON CARTILAGE AND BONE IN TISSUE SECTIONS FROM FETAL RAT HEADS



by

Nicholas Joseph Malinski, Jr.

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
The Requirements for the Degree of
Master of Science

February
1968

## BIOGRAPHY

Nicholas Joseph Malinski, Jr., was born in Chicago, Illinois, on August 15, 1943. In June, 1961 he graduated from St. Benedict High School, Chicago, Illinois.

He entered John Carroll University, University Heights, Ohio in September of 1961 and graduated in June, 1965 with the degree of Bachelor of Science in Biology.

The author began his graduate studies in the Department of Anatomy, Loyola University Stritch School of Medicine in September, 1965. Since July, 1966 he has held the position of Graduate Teaching Assistant in Anatomy at Loyola.

On June 25, 1966, the author married the former Miss Patricia Ann Pedini of Cleveland, Ohio, and on April 1, 1967, the couple became the proud parents of Nicholas III.

Mr. Malinski is a member of the American Institute of Biological Sciences.

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to his advisor, Dr. Robert C. Clawson, for his guidance and encouragement in this investigation. The interest Dr. Clawson showed not only in this investigation but in this investigator will always be remembered.

My thanks also go to Dr. Leslie A. Emmert and Dr. Maurice L'Heureux for their assistance in finishing this work. Their criticism and help proved to be invaluable in the completion of this thesis.

The author wishes to express his gratitude to the staff and students of the Department of Anatomy for their support and never ending availability to give assistance. Special thanks are given to Dr. Lincoln V. Domm for the suggestion which eventually led to this investigation, and much appreciation is extended to Dr. Herbert Barghusen for his advice and help during the critical start of this study.

Finally, I would like to thank my wife, Patricia, for the fine job of typing this thesis and the encouragement which she so generously provided.

TABLE OF CONTENTS

LIST OF TABLES

## LIST OF FIGURES

# INTRODUCTION

Although extensive research has been done on the role of parathyroid hormone in bone resorption, there is a great deal that still remains unknown. In recent years the literature has mirrored the great interest in solving the problem of how parathyroid hormone effects bone resorption. Advances in recent decades are the result of the application of new research tools of which among the most important is the method developed to purify parathyroid hormone (Aurbach and Potts, '67). Armed with a relatively pure parathyroid extract (PTE) the investigator has unlimited vistas for his curiosity. The chemistry of the hormone, the causes for its secretion, its biochemical action and finally the mechanism of its action, are a few of the areas for investigation. Work done in these areas is discussed and brought into perspective in review works by Greep and Talmage ('61), Gaillard and Talmage ('65), and Aurbach and Potts ('67).

This investigation involves a study of the effects PTE has on fixed and fresh frozen, sectioned, fetal rat heads, as demonstrated by various histochemical staining procedures specific for calcium, collagen, and acid mucopolysaccharides of cartilage and bone. Slides of fixed and fresh frozen, sectioned, fetal

rat heads were incubated in solutions of PTE for times of up to 48 hours and then histochemically stained to detect alterations. For purposes of controls, slides were similarly incubated without PTE.

It is the opinion of this investigator that the approach will shed new light on the problem of how PTE effects bone resorption.

## REVIEW OF LITERATURE

Research into the mechanism of bone resorption is centered around the use of purified bovine parathyroid hormone. It is a single chain polypeptide, molecular weight of about 8,500, without any covalent intra-chain cross linkage (Aurbach and Potts, '67). The purified product stimulates both calcium mobilization from bone and phosphaturia in parathyroidectomized animals (Aurbach, '59).

In recent experiments (Potts and Aurbach, '66) evidence has been gathered that indicates of the 80 or so amino acids of the polypeptide chain only 16 are necessary for biological activity. Within this active fragment it has been determined that the methionine, tryptophan, and tyrosine are each critically important for biological activity. Oxidation of the sulfur to the sulfone on a methionine ($R\text{-}S\text{-}CH_3$) $\longrightarrow$ ($R\text{-}SO_2CH_3$) results in loss of biologic activity (Copp, '65). Immunologic activity of the molecule is not markedly altered unless the tyrosine is modified (Potts and Aurbach, '65). These studies show that the regions required for biological and immunologic activity although not identical, share a common sequence of amino acids. This relationship permits immunoassay to detect the hormone in physiologic fluids (Aurbach and Potts, '67).

Secretion of the hormone seems to be primarily determined

by blood calcium levels. Some recent work suggests that magnesium and phosphate also directly influence parathyroid secretion, but this evidence at best only shows an indirect influence upon secretion through their effect on calcium levels in the blood (Aurbach and Potts, '67; Talmage and Taft, '61).

The hormone has been observed to produce two effects both independent of the other, one is calcium mobilization from the skeleton and the other is the production of phosphaturia. The principle physiologic function of the hormone is regulation of calcium in body fluids through resorption of calcium from bone. The phosphaturic effect might be considered "extraskeletal" unrelated to hormonal control of calcium (Aurbach and Potts, '67). Recent identification of thyrocalcitonin (Hirsch et al., '63), a secretion of the thyroid gland, may explain the phosphaturic response since this hormone may itself contribute to the phosphaturic response (Robinson, '65). Thyrocalcitonin is also reported to act on bone (Aliapoulios et al., '66) as an inhibitor of bone resorption (Wallach et al., '67). This evidence would then still leave the physiologic significance of the phosphaturic response unsettled.

There exists three prominent theories of how parathyroid hormone affects bone resorption: (1) the hormone induces enzymatic destruction of bone matrix (Walker et al., '64; Vaes, '65), (2) it influences enzymic production of organic acids or chelators to dissolve bone salt (Firschein et al.,

'58), (3) or it activates biological ion transport systems that might "pump" calcium and/or phosphate from bone into the extracellular fluid (DeLuca et al., '62).

In the first theory, a proteolytic potential of the mature hypertrophic osteocyte which is augmented by PTE, is pointed to as being capable of releasing calcium by destruction of the organic matrix to which calcium is bound (Belanger, '65). This theory is challenged by Bohatirchuk ('63) who observed collagen fibers completely denuded of their calcium crystals, without being destroyed. Bohatirchuk ('66) postulates calciolysis is the initial stage of bone resorption while the second stage is a removal of the denuded matrix.

The second theory concerns what has been called the "acid mechanism theory" of bone resorption (Newman and Newman, '58). Certain experiments have shown that the injection of PTE may lead to increased production by bone of citrate (Firschein et al., '58) or lactate (Borle, '60). This idea is found defective in that stoichiometrically speaking, the increments in production of acid do not seem to account for the amount of calcium mobilized from bone (Aurbach and Potts, '67). In short, more calcium is mobilized than is accounted for in citrate production.

Recently, interest has developed in the third possibility as an outgrowth of experiments showing that PTE <u>in vitro</u> causes rapid effects on calcium and phosphate transport in kidney or liver mitochondria. Under appropriate conditions, the hormone

*in vitro* causes the mitochondria to give up calcium (DeLuca et al., '62) or stimulates phosphate (Sallis et al., '63) potassium and magnesium, (Rasmussen et al., '64) uptake by the mitochondria. The hormone also causes accelerated respiration and pyridine nucleotide oxidation of mitochondrial suspensions (Rasmussen and Ogata, '66). These observations have led to the postulation that these mitochondrial effects by PTE are the basis for its biochemical mechanism (Rasmussen and Ogata, '66). Other studies (Aurbach and Potts, '65), however, indicate that the effects of PTE on mitochondria are produced by other substances, namely other basic proteins, like histone, protamine polylysine and a nonhumeral protein contaminant in crude PTE preparations. Also, since liver mitochondria respond to the PTE, doubt is cast on the results simply because the liver is not recognized as a target organ for the action of parathyroid hormone (Aurbach and Potts, '67).

Basically, all research on the problem of how parathyroid hormone affects bone resorption points to an enzymatic system at the level of the cell or subcellular level which PTE activates. The need for osteoclasts to produce these enzymes is disputed (Bohatirchuk, '63; Belanger et al., '63; Belanger, '65) or the prerequisite that the bone tissue be living to effect calcium removal by PTE is also disputed (Martindale and Heaton, '65; Stern and Raiz, '67). The need for increased acidity to produce an environment in which the necessary enzymes can facilitate bone

resorption can not be overemphasized (Vaes, '65). Whether these enzymes are present in all mineralized tissue living or dead (Fullmer, '66) is disputed, and a further point of contention is whether PTE can activate these enzyme systems in dead tissue (Martindale and Heaton, '65; Stern and Raiz, '67).

Vaes ('65) postulates PTE causes bone cells to secrete lysosomal hydrolases at the resorption sites. Another effect of PTE is to cause a pH shift that favors enzymatic activity of these lysosomal hydrolases. Because of the pH shift bone mineral is more soluble and this leaves the bone matrix denuded and available to the digestive action of the lysosomal hydrolases.

This study is limited to histological observations made on components of bone and cartilage, known to be involved in bone resorption after treatment with PTE. A brief review of literature on the roles of calcium, collagen, and acid mucopolysaccharides in bone formation and resorption will follow.

Calcium.

Calcification or deposition of calcium in the form of an hydroxyapatite ($Ca_3(PO_4)_2$ crystal in the organic matrix is accomplished by precipitation. The precipitation is from the body fluids when concentration is around the supersaturation point and is favored by an alkaline environment. Conversely, resorption is favored by an acidic environment (Ham, '65).

Robinson ('32) has shown large amounts of alkaline phosphatase in areas of calcification. The presence of this enzyme is

thought to produce phosphate (from sugar phosphates) that could initiate calcification. Deposition of calcium is thought to begin in the acid mucopolysaccharides of the organic matrix. An almost sponge-like affinity for calcium precipitates is exhibited by the intercellular substance of cartilage (Ham, '65).

Collagen.

Collagen is the commonest protein in the body. In bone it serves to reinforce the organic matrix. Collagen in bone arises as a secretory product of osteoblasts, and has been thought to serve as a nucleus upon which calcium crystal formation can occur (Ham, '65).

Since collagen is a major component of cartilage and bone matrix, its breakdown must be an intricate part of the resorption mechanism (Lapiere and Gross, '63). Stern and Mechanic ('63) determined from finding hydroxyproline (a component of collagen) in the tissue culture medium that collagen was being removed during active bone resorption.

Collagen breakdown *in vitro* is increased by PTE, (Walker et al., '64; Kaufman et al., '65). Woods and Nichols ('63) found that there is a collagenolytic factor located in bone cells. He postulates that this enzyme is not contained or released in areas of bone matrix synthesis. Stern and Mechanic ('63) also postulates an enzymatic system located in bone tissue capable of degrading collagen. Gaillard ('55) found that in areas of bone resorption aniline blue would stain collagen intensively, evi-

dently due to some breakdown of the collagen.

Acid Mucopolysaccharides.

Acid mucopolysaccharides represent the amorphous intercellular substance of bone and cartilage. They are a secretion of the osteoblasts in the case of bone and chondroblasts in the case of cartilage and occur as carbohydrate polymers with acidic side chains. These acidic side chains can be either simple organic acid groups - COOH - or sulfuric acid groups. An example of an acid mucopolysaccharide containing a simple organic acid side chain is hyaluronic acid; an example of a sulfuric acid group containing acid mucopolysaccharide is chondroitin sulfuric acid of cartilage (Ham, '65).

The acid mucopolysaccharides of bone and cartilage or chondroitin sulfate and hyaluronic acid serve as a cement substance in which collagenic fibers are embedded. The presence of these cementing substances is much greater in cartilage than in bone and for this very reason cartilage can take up more mineral than bone (Ham, '65). Cameron et al., ('67) using the electron microscope, observed calcium being deposited in the amorphous substance of cartilage, but in bone, calcium seemed to be deposited on collagen.

Heller-Steinberg ('51), Engel ('52), and Gaillard ('55) have reported an intensified staining of mucopolysaccharides in areas where bone removal is occurring. Zawich-Ossentiz ('27), Kind ('51), Ruth ('54, '61) have recognized the association of

basophilic islands in osseous tissue undergoing resorption. Engel's ('52) histochemical evidence shows that the increase in the intensity of staining following the administration of PTE is due to a breakdown of bone matrix. Gaillard ('55) reports that a strong basophilic reaction, in the histochemical staining of bone undergoing resorption was found in areas yet to be resorbed indicating the possibility of flow away from the actual resorbing sites.

Metachromasia in tissue sections has been recognized as an index to the presence of acid mucopolysaccharides (Walker, '61), and therefore a loss of metachromasia would indicate a loss of acid mucopolysaccharides.

## MATERIALS AND METHODS

The technique used involved the incubation of fixed and frozen tissue sections from fetal rat heads with solutions of parathyroid extract[1] (PTE), and was designed to study the effect of PTE on components of bone and cartilage known to be involved in bone resorption.

Preparation of Materials.

Pregnant albino Sprague-Dawley rats determined to be an average of 17-1/2 days pregnant were sacrificed by terminal ether anethesia. Fetuses from these pregnant rats were sacrificed by decapitation and the heads were immediately placed in one of the chemical fixatives listed in the table below.

Table 1

Fixatives and Time of Fixation

(1) Neutral Formalin - 48 hours

(2) (F.A.A.) Formaldehyde, Acetic Acid and Absolute Alcohol in proportion of (1-1-18) - 48 hours

(3) Acetone - cold 24 hours

(4) 80% alcohol - cold 24 hours

(5) 1/2 acetone and 1/2 80% alcohol - cold 24 hours

---

[1] The PTE used throughout this investigation was the commercial preparation Paroidin by Parke-Davis.

A freeze technique was also employed in which the fresh rat heads were immediately frozen at -22°C, in the refrigeration unit which houses a Cryo-Cut microtome (American Optical Company). Serial sections were made using the same Cryo-Cut microtome also at a -22°C. These sections were cut at 13 microns and placed on consecutive slides to dry for 15-20 minutes before incubation.

The fixed rat heads were embedded in paraffin and serial sectioned at 6-8 microns. These sections were placed on slides with and without egg albumen adhesive. After drying for a day, the slides were deparaffinized in xylene and hydrated in a decreasing series of alcohols (100% and two changes of 95%) then thoroughly rinsed in distilled water. The slides were then incubated at 37-38°C in 50 ml of one of the solutions given in Table 2.

Table 2

Solutions Used in Incubating Tissues for 48 Hours

| Incubating Solutions | Purpose Used |
|---|---|
| (1) Distilled water (pH adjusted 3.5-7.6) adjusted with hydrochloric acid & ammonium hydroxide | Establish that pH did not cause results attributed to PTE. |
| (2) Double distilled water (pH adjusted 3.5-7.6) adjusted with hydrochloric acid & ammonium hydroxide | Same as above. |
| (3) Physiological saline (Ringer's and Locke's) | Establish that lack of ionic balance did not cause results attributed to PTE. |

Table 2 (Cont'd.)

Solutions Used in Incubating Tissues for 48 Hours

| Incubating Solutions | Purpose Used |
|---|---|
| (4) Potassium phosphate buffered solutions (6.0-7.0 pH) | Establish that buffered pH solutions did not cause results attributed to PTE |
| (5) .5% phenol in $H_2O$ | Establish presence of this chemical in extract does not cause results attributed to PTE. |
| (6) 2.5 molar acetic acid in $H_2O$ (resulting in 2.5 pH) | Deactivate hormone and used alone to show that this acid at this pH does not cause results attributed to PTE. |
| (7) .1 molar hydrogen peroxide in potassium phosphate buffer (pH 7.0) | Deactivate hormone and used alone to show that this combination did not cause results attributed to PTE. |
| (8) Hyaluronidase in sodium phosphate buffer 7.0 pH | Indicate if PTE and hyaluronidase act on the same substance. |

An experimental slide was incubated in 50 ml of one of the above solutions containing .4 ml of PTE (excluding those in the hyaluronidase and phenol studies where no PTE was added). A control slide was incubated just like an experimental slide but there was no PTE in the incubating media.

Following incubation, slides were stained to detect alterations in calcium, collagen, and acid mucopolysaccharides, according to the following brief résumé.

Calcium.

Alizarin Red S in 1% aqueous solution with an adjusted pH

of 6.3-6.5, gave high specificity and good demonstration of calcium salts found in tissue sections (Dahl, '52). The pH is adjusted with a 1/100 dilution of 28% ammonia water. This method resulted in calcium showing up as a crimson lake (Lillie, '54).

Collagen.

Mallory's Triple Connective Tissue Stain resulted in aniline blue staining connective tissue and cartilage; orange G staining blood, myelin and muscle; briebrich scarlet staining the remaining tissue elements; and hematoxylin giving a permanent nuclear stain (Humason, '62). Baker ('58) reported that phosphomolybdic acid treatment before and after staining with aniline blue, results in collagen and no other tissue element being stained by the aniline blue.

Acid Mucopolysaccharides.

Acid mucopolysaccharides, both simple and sulfated were well stained by alcian blue and metachromatic dyes at a low pH by a salt linkage with the acidic groups (Steadman, '50). Pearce ('61) regarded metachromatic material in fixed tissues embedded in paraffin, whose metachromasia is reversible by treatment with a purified testis extract (hyaluronidase) to be of a mucopolysaccharide nature. He specifically claims this reversible metachromasia to be due to either the presence of chondroitin sulfate or hyaluronic acid or a combination of them both. Meyer ('46) stated that only chondroitin sulfate would produce metachromasia.

The procedure used for staining with methylene blue is

briefly related at this time, since it was the only procedure modified as found in the literature. Cleared and hydrated slides were placed in 95% alcohol for two minutes and then in a .025% methylene blue solution in 95% alcohol for ten seconds. After this staining period, the slides were immersed in cold distilled $H_2O$ for a quick rinse. The slides were then dehydrated in two changes of acetone, one change of acetone-xylene, and cleared in xylene. All slides were mounted with Harleco Synthetic Resin (HSR).

Table 3

List of Stains Used and Their Purpose

| Procedure Used | For the Purpose of Studying |
|---|---|
| (1) Mallory Triple C.T. (Humason, '62) | Collagen |
| (2) Alizarin Red S (Water) (Pearse, '61) | Calcium |
| (3) Alcian Blue (Water) alone or with neutral red (Drury and Wallington, '67) | Mucopolysaccharides |
| (4) Toluidine Blue (Water) (Pearse, '61) | Mucopolysaccharides |
| (5) Methylene Blue (Alcohol) (See modification above) | Mucopolysaccharides |
| (6) P.A.S. - Counter stained with hematoxylin or fast green (Humason, '62) | Glycogen Alteration |
| (7) Hematoxylin ($H_2O$) (Humason, '62) | General Histology |
| (8) Eosin Y (95% Alcohol) (Humason, '62) | General Histology |
| (9) Fast Green (95% Alcohol) (Humason, '62) | General Histology |

Staining times and pH of stains were watched closely to insure controls and experimentals differed only in the presence of PTE. Slides were dehydrated in acetone (in metachromasia studies) or alcohols, cleared in xylene and mounted with HSR. Histological investigation was then carried out with particular interest paid to differences in stain intensity and metachromasia. These differences were correlated to fixative, composition of incubating solution, pH of solution, and time of incubation.

Hyaluronidase extraction for 24 or 48 hours in a sodium phosphate buffered solution (pH 7.0) was used in experimental controls to establish the nature of the compound PTE was observed to be removing (Drury and Wallington, '67). Acid hydrolysis by acetic acid (2.5 molar) and oxidation with hydrogen peroxide as reported by Rasmussen ('59) were used to deactivate PTE. His technique was modified by this investigator, in that .4 ml of PTE was dissolved in 50 ml of a 2.5 molar solution of acetic acid and then slides were placed in the resulting mixture for incubation. First it was established that the solution of 2.5 molar acetic acid did not reduce metachromasia. In using oxidation to deactivate the hormone, 0.1 moles of hydrogen peroxide was dissolved in a potassium phosphate buffer pH 6.0-7.0; into 50 ml of this solution .4 ml PTE was dissolved. Finally, slides were placed in the total aforementioned solution for incubation.

## EXPERIMENTAL RESULTS

It was determined after incubating tissues with only .2 ml PTE in 50 ml of incubating solution that this concentration was not sufficient to remove all calcium demonstrable by alizarin red S. Further, after attempting a variety of incubation times, 48 hours was decided upon because it gave maximum results (table 4).

In order to detect alterations in the staining of calcium, collagen, and acid mucopolysaccharides after PTE incubation, attention was focused on endochondral ossification centers in the hard palate, membrane ossification centers around Meckel's cartilage, Meckel's cartilage, and nasal cartilage.

General Observations.

Tissue sections from chemically fixed and frozen, fetal rat heads when incubated in .4 ml PTE in 50 ml of water at 37-38°C for 48 hours showed alterations in staining of calcium, collagen, and acid mucopolysaccharides.

Specific Observations: Calcium.

In controls and experimentals, alizarin red S was used to indicate the presence or absence of calcium. The majority of pH studies were conducted during the initial phase of experimentation on the effect of PTE on calcium (table 5). The results from this section indicate that slides incubated in distilled water, physiological saline (Ringer's or Locke's, solutions of

water at pH 3.5-7.0, or phosphate buffered solutions at pH 6.0-7.0 when they contain .4 ml of PTE per 50 cc of solution, and after they have been incubated at 37-38°C for 48 hours show a complete removal of calcium (figures 1-2).

Collagen.

It was observed in sections exposed to PTE treatment for 48 hours that the aniline blue component of the Mallory's connective tissue stains, stained collagen fibers more deeply than it stained collagen in control sections. Treatment consisted of incubating tissue sections at 37-38°C for 48 hours with .4 ml of PTE in 50 ml of distilled water (table 6; figures 3-4).

Acid Mucopolysaccharides.

To determine any difference in acid mucopolysaccharides after treatment of tissue sections with PTE, the intensity of metachromasia produced in bone and cartilage matrix was used as an indication of altered acid mucopolysaccharides (table 6). In control slides there was observed a very clear-cut metachromasia developed after staining with toluidine blue or our modified methylene blue technique (figures 5-6). In experimentals, metachromasia was either greatly reduced or almost completely absent depending on the fixative used (table 7; figures 11 and 14). In experimental tissue sections stained with alcian blue and neutral red, a decreased staining of bone and cartilage matrix was observed (figures 7-8).

Parathyroid Extract.

Using the altered approach of Rasmussen ('59) it was ob-

served that if .4 ml of PTE was incubated in a 2.5 molar solution of acetic acid thus inactivating the hormone, this solution of PTE did not produce a decrease in metachromasia when slides were incubated in this medium (figure 6). No decrease in metachromasia was observed in slides incubated with PTE inactivated by exposure to hydrogen peroxide oxidation. Slides incubated in .5% phenol for times up to 48 hours did not show alteration in calcium comparable to that produced by PTE.

Fixation.

The results of this investigation indicate that the type of fixation has no noticeable effect on the action of PTE. The only difference noted was that in sections obtained from frozen specimens, when exposed to PTE treatment, the metachromasia was observed as tiny sand-like granules in the cartilage matrix, while that in fixed tissues was not (figures 13-14). There was a great decrease in metachromasia and the general appearance of the matrix in frozen tissue sections after incubation with PTE was of a broken down nature. By "broken down," reference is made to the observation that the walls of lacunae were not discernible (figure 13).

Incubating Solutions.

The purpose of this study was to determine the best solution to use as a general incubating media. Since neither the pH of the solution resulting from mixing .4 ml of PTE in 50 ml of water nor the pH of the water itself produced results that the

extract in water produced, distilled water could be used for incubation (table 5). Distilled water was therefore chosen as the solvent for PTE, so that results could be attributed only to the PTE and not to the solvent used.

Hyaluronidase Extraction.

Hyaluronidase extraction was used to pin down the specific acid mucopolysaccharide on which PTE was acting. After treatment with hyaluronidase, it was noted that the same type of metachromasia loss, although not quite as great, was produced as reported previously using PTE. Using both treatments on tissues fixed in formaldehyde containing fixatives, one after the other, a greater loss of metachromasia was observed than with either treatment alone, suggesting similar action on what seems to be chondroitin sulfate (figures 10 and 12).

## DISCUSSION

The results of this work lend support to the contention that PTE can remove calcium from dead tissue (Martindale and Heaton, '65). The method by which this removal is effected could possibly be due to an activation of the enzymatic system described by Vaes ('65).

Almost all research concerning bone resorption has been carried out using *in vivo* and *in vitro* techniques. Parathyroid extract was permitted to act on living tissue, and the effect of PTE was observed after histological preparation of that tissue. Since the approach used in this investigation, differed from those of other investigators, in that PTE was permitted to act on tissues already histologically prepared, an examination of our methods will follow.

The first point that needed to be established, was that results from the technique used were attributable to the biologically active portion of the PTE preparation and not some other component of the extract. It was established that the .5% phenol in the extract was not able to produce results brought about by PTE. Incubating tissue sections in a .5% phenol solution produced control-like results (figure 1). The methods outlined by Rasmussen ('59) for deactivating PTE were modified in order to establish that results were due to an active hormone. When this

oxidized or hydrolyzed hormonal preparation was used, metachromasia was produced as if the hormone was not present in the incubating solution (figure 6). It was found that incubating slides in .1 molar hydrogen peroxide in a phosphate buffer pH 7.0 caused the tissue sections incubating with the deactivated hormone, to detach from the slides in some cases. An explanation for this could be that the incubating solution was strong enough to oxidize protein elements holding the tissue sections to the slides resulting in the sections detaching. This detaching occurred only when using oxidation deactivation and in no other procedures which would indicate the hormone played no role in the detachment of the tissue section.

A variety of fixatives were employed (table 7) and none of the chemical fixatives used seemed to vary the activity of PTE on the metachromatic components of bone and cartilage, although a decrease in background staining was observed in fixatives not containing formaldehyde. This is attributed to the effect of formaldehyde on amino groups, rendering them slightly acidic and therefore basophilic in regard to staining. These amino groups would then take up more of basic dyes. This is the only point of variance in pH that could account for an increased basophilia of tissue elements since the pH of all stains used were watched closely to keep them at the prescribed staining range for maximum results. Sections from frozen prepared rat heads produced the most interesting results. After PTE treatment of sections

from frozen prepared fetal rat heads, a matrix break up was observed most evident in the cartilage matrix (figure 13). Metachromasia in these sections was observed as tiny sand-like granules (which are not clearly shown in figure 13), further indicating a break up of matrix. This observed break up would also indicate a collagenolytic ability in the PTE, or the ability of the extract to liberate the lytic agent from the hypertrophic osteocytes as described by Belanger et al., ('63), or some component of the matrix.

The results as reported can therefore he attributed to the biologically active portion of the parathyroid extract and that neither fixation nor the incubating solutions used are modifying factors as far as can be determined.

That PTE effects removal of calcium from bone is a long established fact. These results indicate that calcium is liberated into the incubating media, from the ossification center as PTE acts on the acid mucopolysaccharides of that center. A determination of what specific acid mucopolysaccharide PTE may act on necessitates a brief résumé of the literature on acid mucopolysaccharides. Hyaluronidase extraction has been shown to cause a decrease or the removal of metachromasia produced by chondroitin sulfate or hyaluronic acid or a combination of them both (Pearse, '61). Meyer ('46) states that only chondroitin sulfate causes this reversible metachromasia. Ham ('65) claims that chondroitin sulfate is the only acid mucopolysaccharide

that is not washed out of cartilage and bone in fixation. Finally, Engel ('52) relates that chondroitin sulfate and other like acid mucopolysaccharides are the cementing substances between calcium and the organic matrix. This short review of pertinent literature tends to indicate that PTE was acting on chondroitin sulfate in the metachromasia studies of this experiment.

Ham ('65) reports that bone and cartilage contain elements for their own destruction. If this destruction system is at the enzyme level as reported by Vaes ('65), might not PTE trigger this mechanism even in fixed tissues (Martindale and Heaton, '65) where activity can occur but not be mediated by any living components?

If one conceives of acid mucopolysaccharides as bathing collagen fibers, and calcium crystals as being impregnated in this amorphous acid mucopolysaccharide substance as well as on the surface of collagen fibers (Ham, '65), the following theory of bone resorption is indicated by this work. The primary effect of PTE would be on the acid mucopolysaccharide amorphous substance (specifically on chondroitin sulfate) dissolving or depolymerizing it away by methods undetermined at this time. After the dissolution of this amorphous cementing substance, calcium would be freed. The next problem would be a need for degrading collagen. The observations reported in this work on fresh frozen sections after PTE treatment, indicated that PTE

either acted on the collagen or caused something else to act on it resulting in what was called a "broken down" nature. This possible theory of bone resorption, i.e., PTE acts on chondroitin sulfate to remove calcium from bone and then by itself or by causing something else to, degrades collagen is well within the realm of present thought to be probable and represents only the addition of what PTE may primarily act on to produce calcium removal.

## SUMMARY AND CONCLUSIONS

A technique was used in which fixed and frozen tissue sections from fetal Sprague-Dawley albino rats averaging 17-1/2 days fetal age, were incubated in .8% solutions of PTE. The following results are based on over 750 slides from fetal rats of 20 litters.

1. Slides of fixed and frozen sectioned fetal rat heads when incubated with .4 ml of PTE in 50 cc distilled water (.8%) show alterations in the staining of calcium, collagen, and acid mucopolysaccharides.

2. The alterations reported are a loss of histochemically demonstrable calcium, a loss of metachromasia produced by acid mucopolysaccharides, and a more intense staining of collagen; all were observed after incubation with PTE. These alterations correspond with three phases of bone resorption, i.e., (1) the depolymerization of the acid mucopolysaccharides, (2) the removal of calcium, and (3) the degradation of collagen.

3. The alterations have been attributed to the biologically active portion of the extract.

4. Results were not grossly affected by different methods of fixation.

5. The primary action of PTE seems to be the removal of the cementing substance between calcium and the organic matrix. Based on the literature, this substance is thought to be chondroitin sulfate.

## LITERATURE CITED


Aliapoulios, M.A., P. Goldhaber and P. Marison 1966 Thyrocalcitonin inhibition of bone resorption induced by parathyroid hormone in tissue culture. Sci., 151: 330-331.

Aurbach, G.D. 1959 Isolation of parathyroid hormone after extraction with phenol. J. Biol. Chem., 234: 3179.

Aurbach, G.D. and J.T. Potts, Jr. 1965 Effects of parathyroid hormone and other proteins *in vitro* on mitochondrial metabolism. Bio. Chem. and Biophys. Res. Commun., 20: 592.

Aurbach, G.D. and J.T. Potts, Jr. 1967 Parathyroid hormone. Amer. J. Med., 42: 1-8.

Baker, J.R. 1958 Principles of Biological Technique. John Wiley & Co., New York.

Barnicot, N.A. 1948 The local action of the parathyroid and other tissues on bone in intracerebral grafts. J. Anat. (Lond.), 82: 233.

Bates, W.K., J. McGawen and R.V. Talmage 1962 Influence of the parathyroid on plasma hydroxyproline level. Endocrin., 71: 185-195.

Belanger, L.F., J. Robichon, B.B. Migicovsky, H. Copp and J. Vincent 1963 Resorption without osteoclasts (osteolysis). Symposium on mechanisms of hard tissue destruction. Reidar F. Sognnaes, Ed., A.A.A.S., Washington, 531-553.

Belanger, L.F. 1965 Osteolysis: An outlook on its mechanism and causation. In: The Parathyroid Gland, Ultrastructure, Secretion and Function. Ed. Gaillard, P.G.; Talmage, R.V., and Budy, A.M., Chicago. University of Chicago.

Bohatirchuk, F. 1963 A study of bone resorption and osteoclast problem by stain historadiography. Am. J. Anat., 113: 113.

_____ 1966 Calciolysis as the initial stage of bone resorption - a stain historadiographic study. Amer. J. Med., 41: 836-846.

Borle, A.B., N. Nichols and G. Nichols 1960 Metabolic studies of bone *in vitro* II. The metabolic patterns of accretion and resorption. J. Biol. Chem., 235: 1211.

Bronner, F. 1961 Parathyroid effects on sulfate metabolism: interrelations with calcium. In: R.O. Greep and R.V. Talmage (Eds.), The Parathyroids, 123-38. Springfield, Illinois, Charles C. Thomas.

Cameron, D.A., H.A. Paschall and R.A. Robinson 1967 Changes in the fine structure of bone cells after the administration of parathyroid extract. J. Cell Biol., 33: 1-13.

Chang, H.Y. 1951 Grafts of parathyroid and other tissues to bone. Anat. Rec., 111: 23.

Collip, J.B. 1925 Extraction of a parathyroid hormone which will prevent or control parathyroid tetany and which regulates levels of blood calcium. J. Biol. Chem., 63: 396.

Copp, D.H. 1965 The Parathyroid Glands in T.C. Ruch and H.D. Patten (Eds.), Physiology and Biophysics, W.B. Saunders Co., Phila., 1167-1172.

Dahl, L.K. 1952 A simple and sensitive histochemical method for calcium. Proceedings of the Society for Experimental Biology and Medicine., 80: 474-479.

DeLuca, H.F., G.W. Engstrom and H. Rasmussen 1962 The action of vitamin D and parathyroid hormone *in vitro* on calcium uptake and release by kidney mitochondria. Proc. Nat. Acad. Sc., 48: 1604-1609.

Drury, R.A.B. and E.A. Wallington 1967 Carlton's Histological Technique Fourth Edition. New York. 212-218.

Engel, M.B. 1952 Mobilization of mucoprotein by PTH. Arch. of Path., 53: 339-351.

Firschein, H., G. Martin, B.J. Mueryan, B. Strates and W.F. Neuman 1958 Concerning the mechanism of action of parathyroid hormone. I. Ion gradients, J. Amer. Chem. Soc., 80: 1619-23.

Fullmer, H.M. 1966 Enzymes in mineralized tissues. Clin. Orth. and Relat. Res., 48: 285-295.

Gaillard, P.J. 1955 Parathyroid gland tissue and bone *in vitro* (1) Exper. Cell Res. Suppl. 3, 154-169.

Gaillard, P.J. and R.V. Talmage 1965 The Parathyroid Glands. University of Chicago, Chicago.

Goldhaber, P. 1963 Some chemical factors influencing bone resorption in tissue culture. In: R.F. Sognnanes (Ed.) Mechanisms of Hard Tissue Destruction, 600-36. Washington, D.C., American Assoc. for the Adv. of Sci.

Greep, R.O. and R.V. Talmage 1961 The Parathyroids. Charles C. Thomas, Springfield, Illinois.

Ham, A.W. 1965 Histology, J.B. Lippincott, fifth edition. Philadelphia.

Heller, M., F.C. McLean and W. Bloom 1950 Cellular transformations in mammalian bones induced by parathyroid extract. The Amer. Journ. Anatomy, 87: #3 315-337.

Heller-Steinberg, M. 1951 Ground substance, bone salts, and cellular activity in bone formation and destruction. The Amer. Journ. Anatomy, 89: 347-79.

Hirsch, P.F., P.L. Munson and G.F. Gauthier 1963 Thyrocalcitonin Endocrinology 73; 244.

Humason, G.L. 1962 Animal Tissue Techniques. W.H. Freeman Co., San Francisco. 146-48.

Kaufman, E.J., M.J. Glimcher, G.L. Mechanic and P. Goldhaber 1965 Collagenolytic activity during active bone resorption in tissue culture. Proc. Soc. Exptl. Biol. and Med., 120: 632.

Kenny, A.D. 1960 Citric Acid Production by Bone in R.O. Greep and R.V. Talmage (Eds.) The Parathyroids. Springfield, Illinois. Charles C. Thomas, 275-312.

Kind, H. 1951 Studien sur frage der osteolyse. Histologische und chemische untersuchungen an experimentellen frakturen und transplataten. Biet. Path. Anat., 111: 283-312.

Lapiere, C.M. and J. Gross 1963 Animal Collagenase and Collagen Metabolism. Symposium on Mechanism of Hard Tissue Destruction. Ed. by Reidar F. Sognnaes, A.A.A.S., Washington, D.C.

Lillie, R.D. 1954 Histopathologic Technique and Practical Histochemistry. McGraw Hill. New York. 263.

Martindale, L. and F.W. Heaton 1965 The relation between skeletal and extracellular fluid magnesium *in vitro*. Biochem. J., 97: 440-443.

Mecca, C.E., G.R. Martin and P. Goldhaber 1963 Alteration of bone metabolism in tissue culture in response to parathyroid extract. Proc. Soc. Exptl. Biol. Med., 113: 538-540.

Meyer, K. 1946 The biological significance of hyaluronic acid and hyaluronidase. Physiological Reviews, 27: 335.

Newman, W.F. and M.W. Newman 1958 The chemical dynamics of bone mineral. University of Chicago Press. Chicago.

Nichols, G. and J.F. Woods 1963 Collagenolytic activity in mammalian bone. Science, 142: 386.

Nordin, B.E.C. 1957 The solubility of powdered bone. J. Biol. Chem., 227: 551-563.

Ossi, L. 1967 Parathyroid-induced changes in collagen and calcium metabolism *in vivo*. Endocrinology, 80: 815-24.

Pearse, A.G.E. 1961 Histochemistry Theoretical and Applied. Little, Brown and Company. Boston. 248-261.

Potts, Jr., J.T. and G.D. Aurbach 1965 The Chemistry of Parathyroid Hormone in the Parathyroid Glands. Ed. by P. Gaillard, R. Talmage and A. Budy. Univ. of Chicago. Chicago. 53-67.

Potts, Jr., J.T., G.D. Aurbach and L.M. Sherwood 1966 Parathyroid hormone: chemical properties and structural requirements for biological and immunological activity. Rec. Prog. Horm. Res., 22: 101-151.

Rasmussen, H. 1959 The stability *in vitro* of parathomone B. J. Biol. Chem., 234: #3 547-550.

Rasmussen, H. and L.C. Craig 1959 Purification of parathyroid hormone. J. Amer. Chem. Soc., 81: 5003.

_____ 1962 The Parathyroid polypeptides. Recent Progr. Hormone Res., 18: 269-289.

Rasmussen, H., J. Fischer and C. Arnaud 1964 Parathyroid hormone, ion exchange, and mitochondrial swelling. Proc. Nat. Acad. Sc., 52: 1198.

Rasmussen, H. and E. Ogata 1966 Parathyroid hormone and the reactions of mitochondria to cations. Biochemistry, 5: 733.

Robinson, R. 1932 The Significance of Phosphate Esters in Metabolism. New York Univ. Press. New York.

Robinson, R.H. 1965 Electron microscopy of cartilage and bone matrix. J. Biophys. and Biochem. Cytol. Suppl. 2. 253.

Ruth, E.B. 1954 Further observations on histological evidence of osseous tissue resorption. Anat. Rec., 118: 347-abs.

_____ 1961 Basophilic islands in osseous tissue and their relation to resorption. Anat. Rec., 140: 307-20.

Sallis, J.D., H.F. DeLuca and H. Rasmussen 1963 Parathyroid hormone dependent uptake of inorganic phosphate by mitochondria. J. Bio. Chem., 238: 4098-4102.

Stern, B. and G.L. Mechanic 1963 The resorption of bone collagen in tissue culture. Biochem. and Biophy. Res. Commun., 13: #2 139-142.

Stern, P.H. and L.G. Raiz 1967 An analysis of the role of serum in parathyroid hormone - induced bone resorption in tissue culture. Exper. Cell. Res., 46: 106-120.

Steadman, H.F. 1950 Staining of acid mucopolysaccharides. Quart. J. Micro. Sci., 91: 477.

Talmage, R.V., S.B. Doty, C.W. Cooper, C. Yates and J. Neuenschwander 1965 Cytological and Biochemical Changes resulting from Fluctuations in Endogenous Parathyroid Hormone Levels. In: The Parathyroid Glands. Ed. by P. Gaillard, R. Talmage and A. Budy. Univ. of Chicago Press. Chicago. 107-124.

Talmage, R.V. and R.J. Taft 1961 The problem of the control of parathyroid secretion. In: R.O. Greep and R.V. Talmage. Ed., The Parathyroids. Charles C. Thomas, Springfield, Illinois.

Tenenhouse, A., R. Nier and H. Rasmussen 1966 Parathyroid hormone and bone mobilization in vitro. J. of Biol. Chem., 241: #6 1314-1318.

Thompson, D.L. and L.I. Pugsley 1932 On the mechanism of PTE action. Amer. J. Physiol., 102: 350-354.

Vaes, G. 1965 Excretion of acid and of lysosomal hydrolytic enzymes during bone resorption induced in tissue culture by parathyroid extract (PTE). Experimental Cell Res., 39: 47074.

Walker, B.E. 1961 The association of mucopolysaccharides with morphogenesis of the palate and other structures in mouse embryos. J. of Embroyol. and Exp. Morphology, 9: 22-31.

Walker, D.G., C.M. Lapiere and J. Gross 1964 A collagenolytic factor in rat bone promoted by parathyroid extract. Biochem. and Biophys. Res. Commun., 15: #5 401.

Wallach, S., A. Chausmer, R. Mittleman and A. Demich 1967 *In vivo* inhibition of bone resorption by thyrocalcitonin Endocrin., 80: 61-64.

Woods, J.F. and G. Nichols, Jr. 1963 Collagenolytic activity in mammalian bone. Science, 142: 386-387.

_____ 1965 Collagenolytic activity in rat bone cells. J. Cell Biol., 26: 747.

Zawisch-Ossentiz, C. 1927 Uber inselin von basophiler substanz in den diaphysen langer rohrenknochen. Z. Mikr.-Anat. Forsch., 10: 472-526.

Table 4

Effects of Varying the Length of Incubation and the Concentration of PTE on Removal of Calcium

| Slides Used | Conc. in 50 ml $H_2O$ | Time of Incubation | Observations on the Alizarin Red S Demonstration of Calcium after Incubation with PTE |
|---|---|---|---|
| 10 | .2 ml | 6 hrs. | no determinable calcium removal |
| 14 | .2 ml | 24 hrs. | slight calcium removal |
| 11 | .4 ml | 24 hrs. | slight amounts still present |
| 175 | .4 ml | 48 hrs. | no demonstrable calcium |
| 15 | .4 ml | 96 hrs. | no improvement on 48 hr. results |

Table 5

pH Studies Incorporating

Results From All Fixatives Used

| pH of Incubating Solution | Effect on Calcium No. Slides | Effect on Metachromasia No. Slides |
|---|---|---|
| 2.5 pH | 5 removes CA | 19 None |
| 4.1 | 7 None | -- ---- |
| 5.0 | 21 None | -- ---- |
| 5.5 | 108 None | 265 None |
| 4.5 | 6 None | -- ---- |
| 5.5 | 10 None | -- ---- |
| 6.0 | 11 None | 8 None |
| 7.4 | 7 effects calcium removal | 15 None * |

* Causes tissue to detach from slides in some cases

Table 6

## Table of Stains Used and Results of Microscopic Examination of Control and Experimental Slides

| Stain Used | Purpose of Stain Used | Tot. No. of Slides Stained | Histologic Difference from Controls |
|---|---|---|---|
| Alizarin | detect change in calcium | 175 + | absence of staining |
| Alcian Blue& Neutral Red | detect change in acid Mucopoly. | 55 * | decreased intensity of staining |
| Periodic Acid Shifts Fast Green | detect change in glycogen | 20 * | no difference |
| Methylene Blue | detect change in acid Mucopoly. | 180 + | great decrease in metachromasia |
| Toluidine Blue | detect change in acid Mucopoly. | 125 + | great decrease in metachromasia |
| Hematoxylin Eosin Y | general histology | 50 * | decrease intensity |
| Mallory's Triple Connective Tissue | detect alteration in collagen | 25 * | increase intensity aniline staining |
| Fast Green | general background histology | 105 * | decrease intensity of stain |

* 23 fetal rat heads provided tissue for these staining procedures

\+ All 56 fetal rat heads used (see Table 7 ) provided tissue sections for these staining procedures

Table 7

Summary of Results Based on Fixatives Used

| Fixative | No. of Fetal Rat Heads | Total No. of Slides Prod. and Stained | After Treatment with PTE: Observ. on Residual Metachromasia No. Slides | | After Treatment with PTE: Observ. on Residual Calcium No. Slides | |
|---|---|---|---|---|---|---|
| F.A.A. (1-1-18) | 15 * | 325 | 106 | Slight | 73 | None |
| Neutral Formalin | 15 * | 245 | 93 | Slight | 65 | None |
| Acetone | 8 + | 60 | 25 | Slight | 8 | None |
| 80% Alcohol | 8 + | 60 | 21 | Slight | 10 | None |
| 1/2 80% Alcohol 1/2 Acetone | 8 + | 60 | 30 | Slight | 9 | None |
| Frozen | 3 + | 50 | 30 | Slight | 10 | None |

* Intermixed from sixteen different mothers

\+ Intermixed from four different mothers

## PLATE I

### EXPLANATION OF FIGURES

1 Tissue section of hard palate incubated in 50 ml of distilled water pH 5.5 for 48 hours and stained with alizarin red S. Tissue was fixed in F.A.A. This photomicrograph is representative of results obtained in all control situations stained to show presence of calcium. Note red alizarin lake formation. X100.

2 Tissue section of hard palate incubated in 50 ml of distilled water pH 5.5 containing .4 ml parathyroid extract (PTE) for 48 hours and stained with alizarin red S. This photomicrograph is representative of results obtained in all experimental situations stained to show effect of PTE on calcium in membrane and endochondral ossification centers. Note lack of red alizarin lake formation. X100.

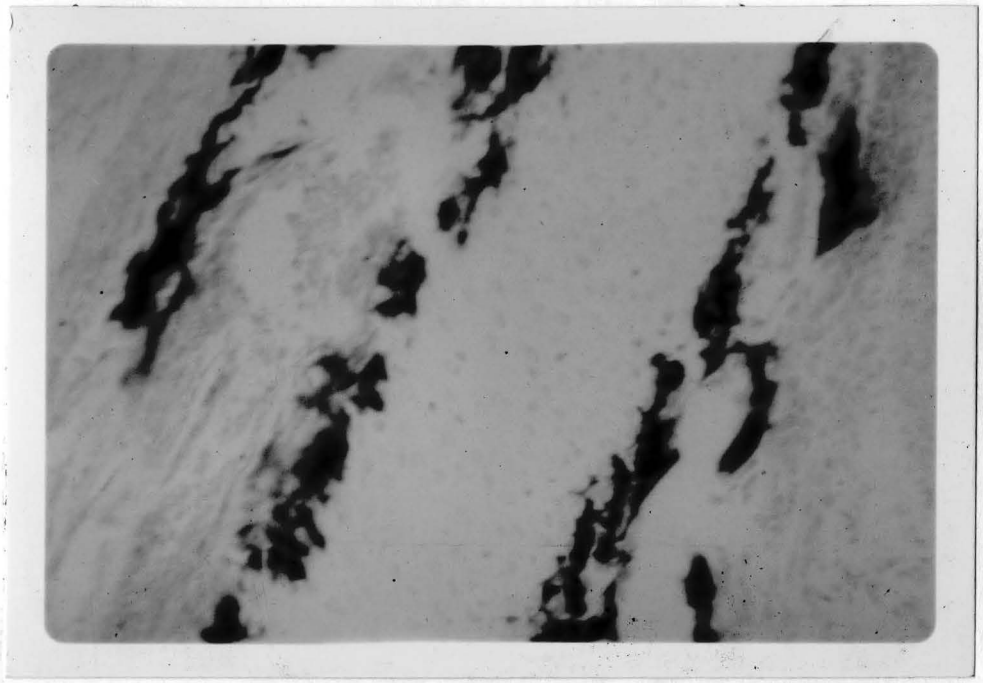

FIGURE 1

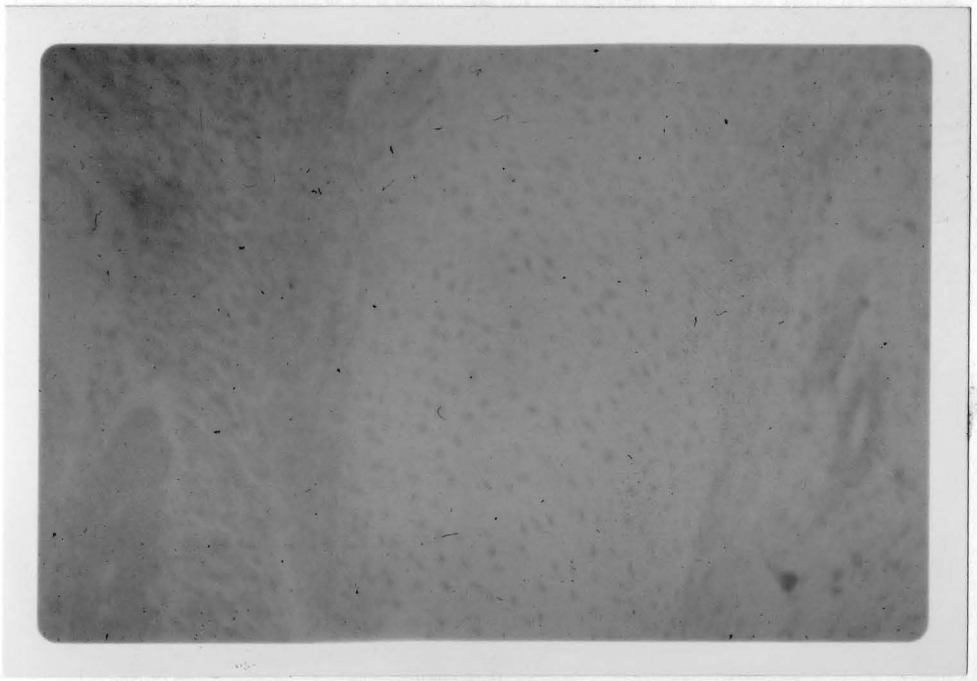

FIGURE 2

## PLATE II

## EXPLANATION OF FIGURES

3 Tissue section of Meckel's cartilage incubated in 50 ml of distilled water pH 5.5 for 48 hours and stained with the Mallory's Triple Connective Tissue Stain. The tissue was fixed in neutral formalin. Note slight blue staining of collagen. X100.

4 Tissue section of Meckel's cartilage incubated in 50 ml of distilled water pH 5.5 containing .4 ml PTE for 48 hours and stained with the Mallory's Triple Connective Tissue Stain. Tissue was fixed in neutral formalin. Note intense staining of collagen. X100.

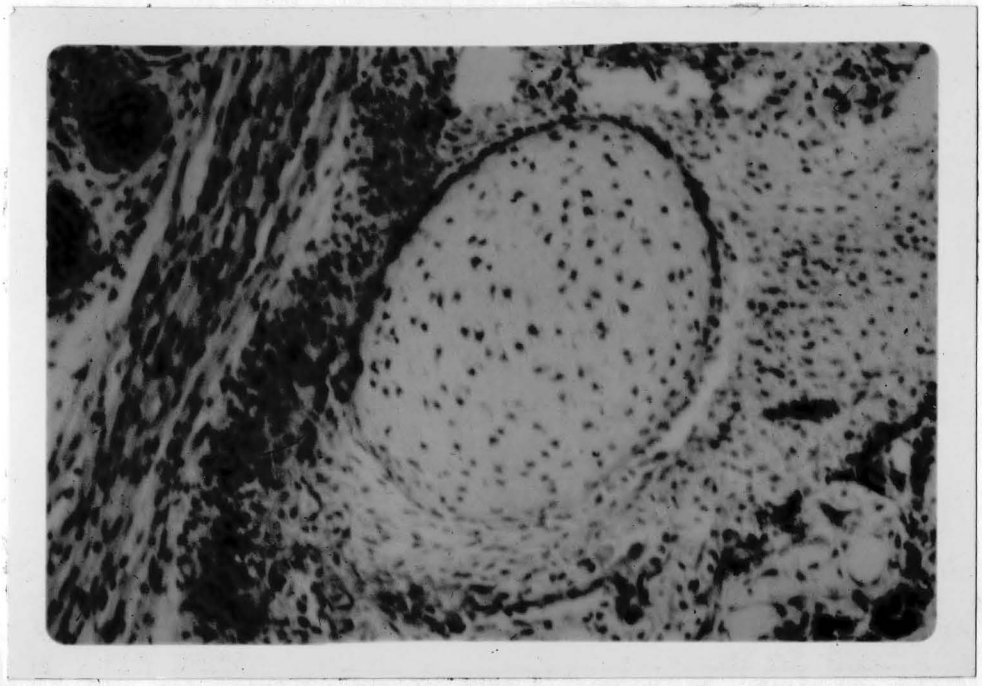

FIGURE 3

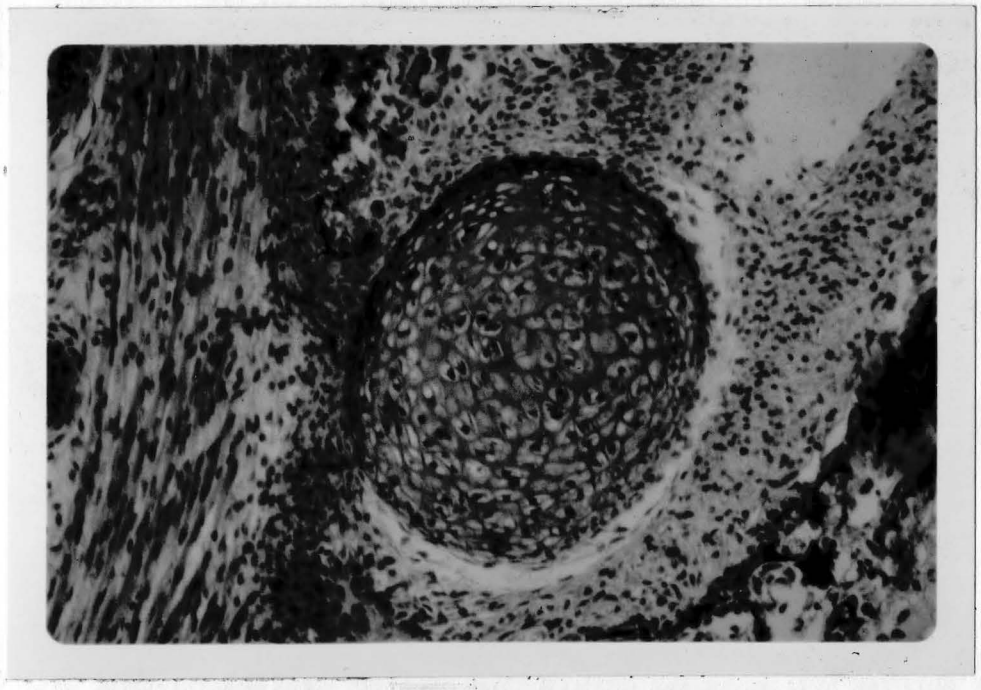

FIGURE 4

## PLATE III

### EXPLANATION OF FIGURES

5 Tissue section of Meckel's cartilage incubated in 50 ml of distilled water pH 5.5 for 48 hours and stained with the metachromatic dye toluidine blue. Tissue was fixed in 80% alcohol. This photomicrograph is indicative of all slides stained with toluidine blue under control situations or conditions described in the text as "control like". Note depth of staining. X100.

6 Tissue section of Meckel's cartilage incubated in 50 ml of a 2.5 m solution of acetic acid for 48 hours containing .4 ml of PTE and stained with methylene blue. Tissue was fixed in 80% alcohol. This photomicrograph is also representative of control results when slides are stained with methylene blue. Note depth of staining. Compare with figure 11. X100.

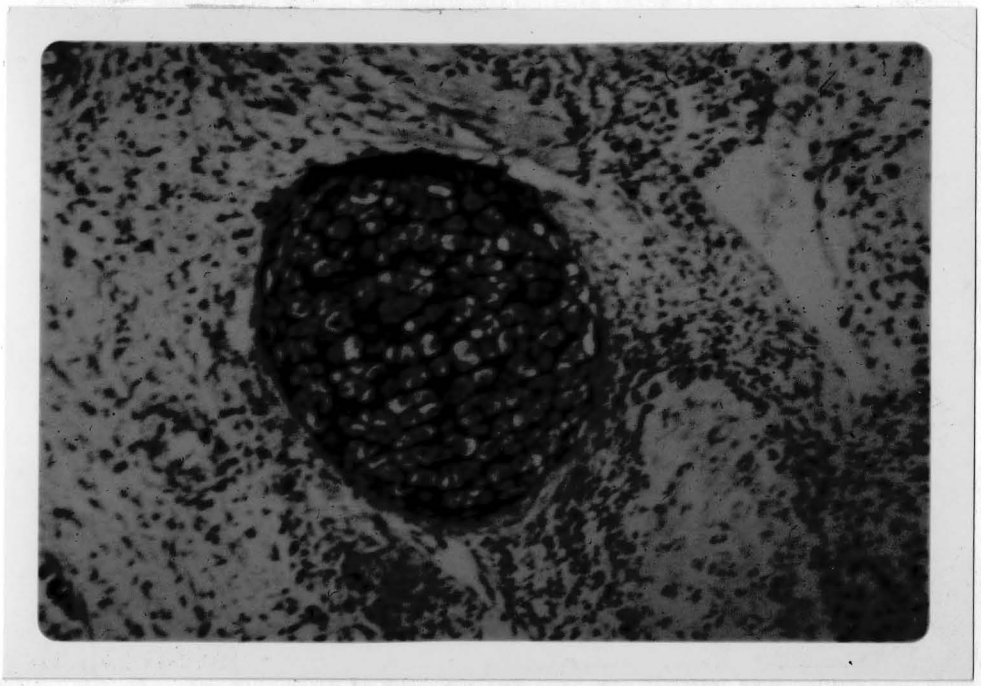

FIGURE 5

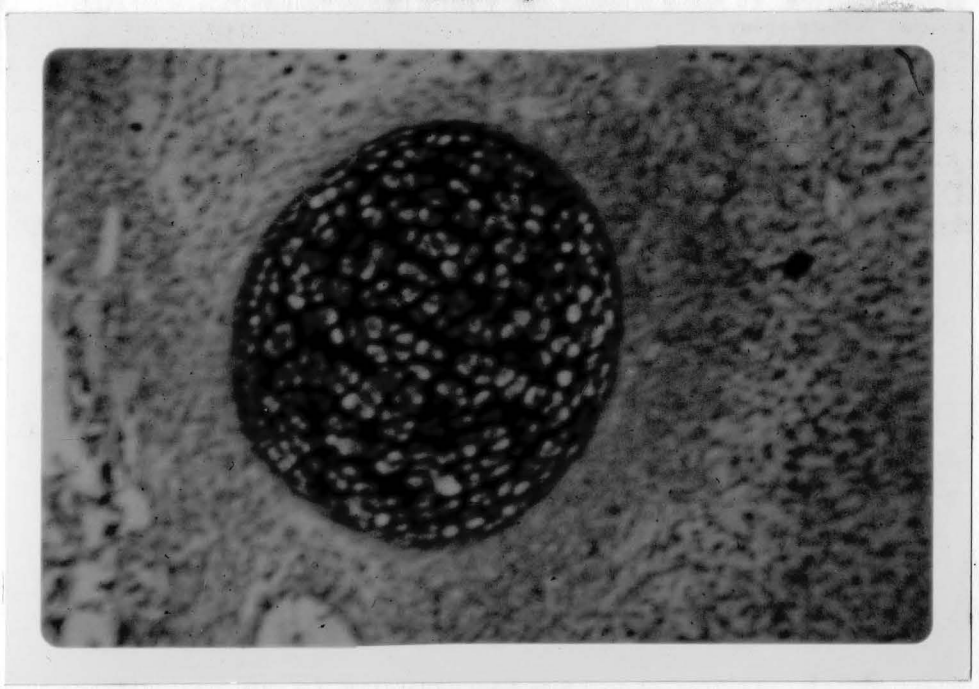

FIGURE 6

## PLATE IV

### EXPLANATION OF FIGURES

7 Tissue section of nasal cartilage incubated in 50 ml of distilled water pH 5.5 for 48 hours and stained with alcian blue and neutral red. Tissue was fixed in F.A.A. This photomicrograph is representative of control results when slides are stained with alcian blue and neutral red. Note staining of alcian blue. X100.

8 Tissue section of nasal cartilage incubated in 50 ml of distilled water pH 5.5 containing .4 ml PTE for 48 hours and stained with alcian blue and neutral red. Tissue was fixed in F.A.A. This photomicrograph is representative of experimental results when slides are stained with alcian blue and neutral red. Note lighter alcian blue staining when compared to figure 7. X100.

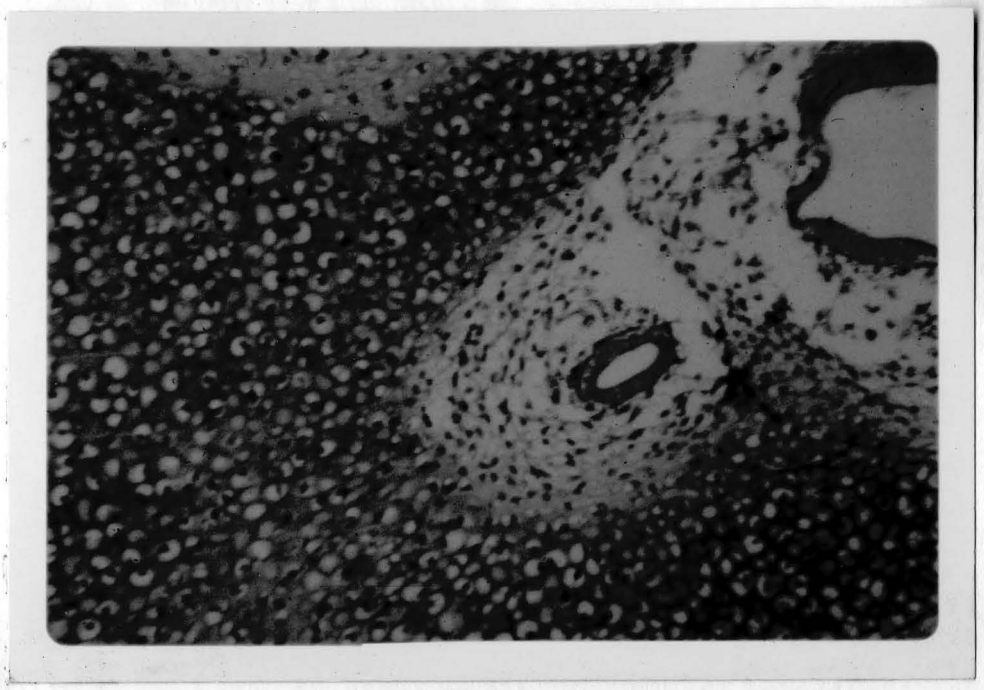

FIGURE 7

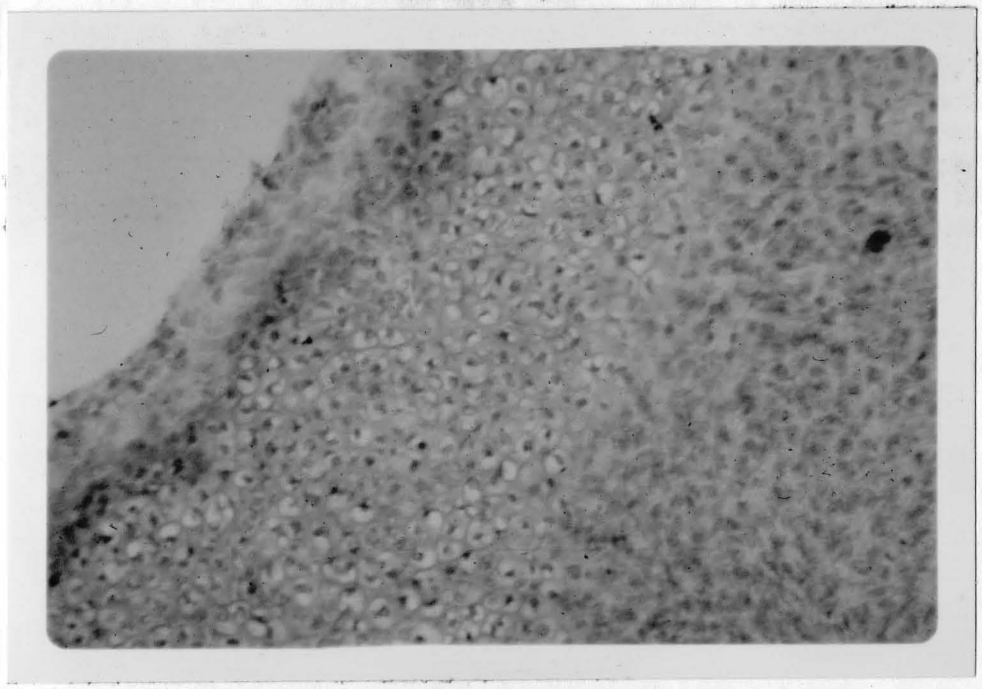

FIGURE 8

## PLATE V

## EXPLANATION OF FIGURES

9 Tissue section of nasal cartilage incubated in 50 ml of a .1 m concentration of hydrogen peroxide in a potassium phosphate buffer pH 7.0 for 48 hours and stained with toluidine blue. Tissue was fixed in 80% alcohol. Note no loss of metachromasia. X100.

10 Tissue section of nasal cartilage incubated in 50 ml of a phosphate buffer pH 6.9 containing hyaluronidase for 48 hours and stained with toluidine blue. Tissue was fixed in F.A.A. Note lighter staining when compared to figure 9. X100.

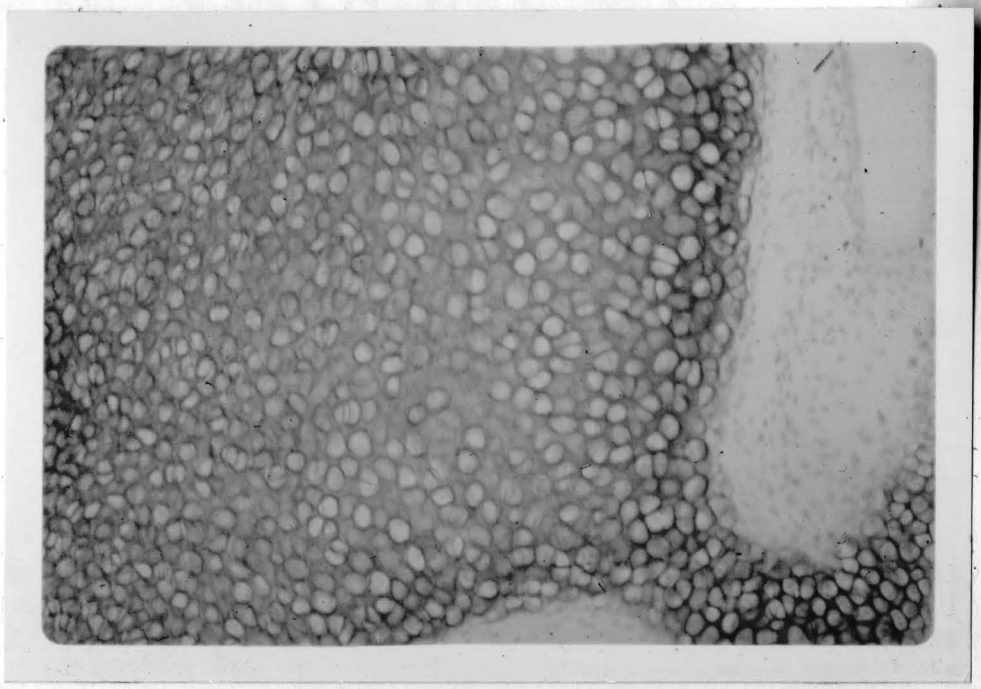

FIGURE 9

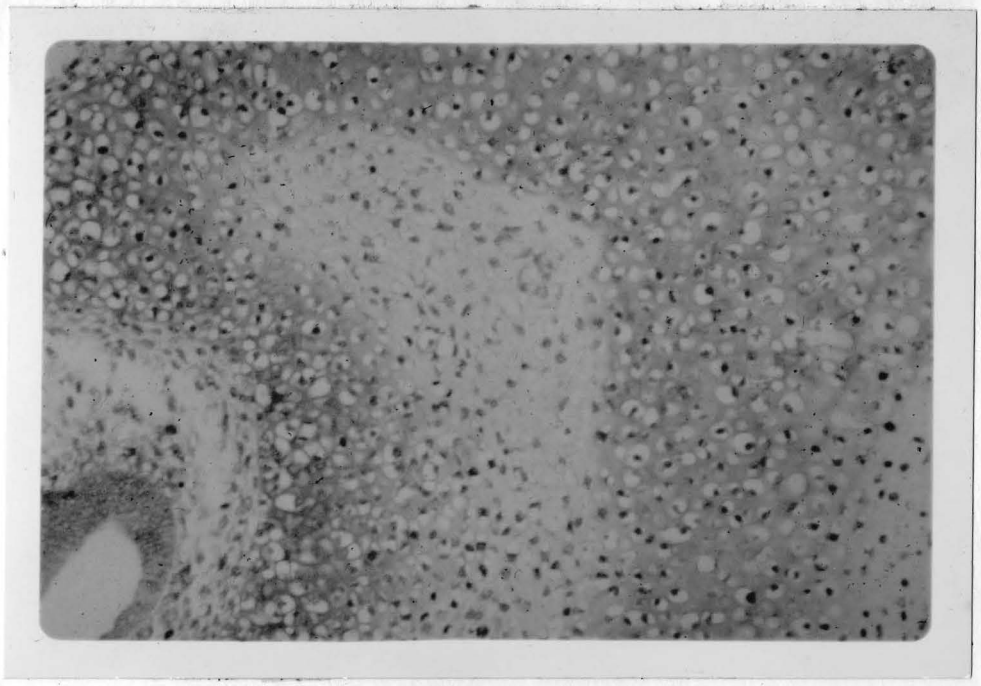

FIGURE 10

## PLATE VI

## EXPLANATION OF FIGURES

11 Tissue section of Meckel's cartilage incubated in 50 ml of distilled water pH 5.5 containing .4 ml PTE for 48 hours and stained with methylene blue. Tissue was fixed in 80% alcohol. This photomicrograph is representative of experimental results when slides are stained with methylene blue. Note almost a complete lack of staining. Compare with figure 6. X100.

12 Tissue section of nasal cartilage incubated in 50 ml of a phosphate buffer pH 6.9 containing hyaluronidase for 48 hours and then in distilled water (50 ml) pH 5.5 containing .4 ml of PTE for 48 hours and finally stained with toluidine blue. Tissue was fixed in F.A.A. Note almost a complete lack of staining, compare to figures 10 and 14. X100.

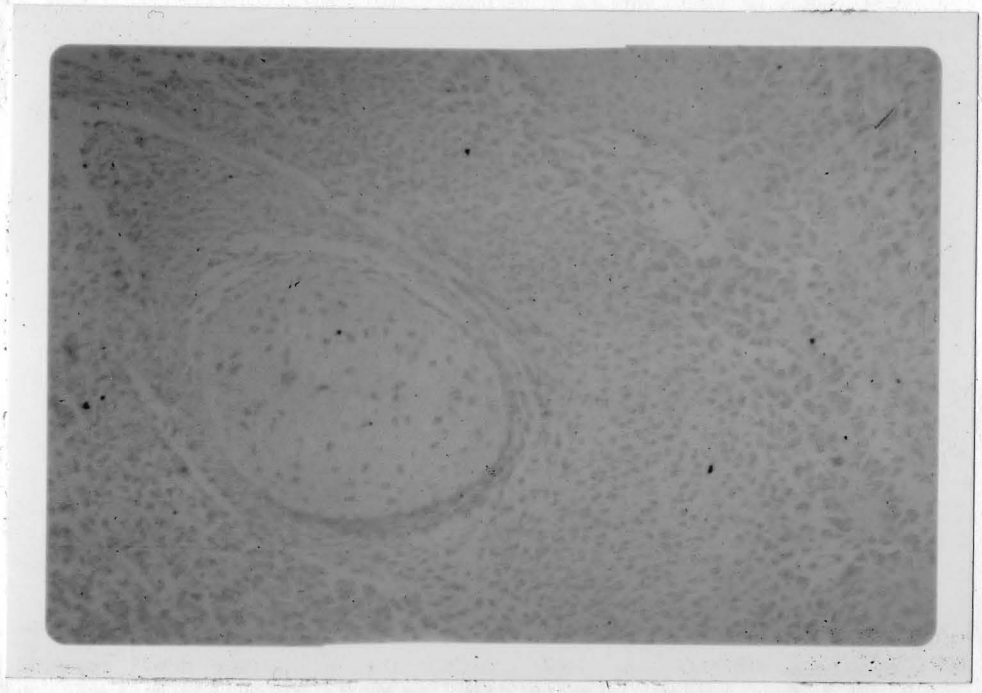

FIGURE 11

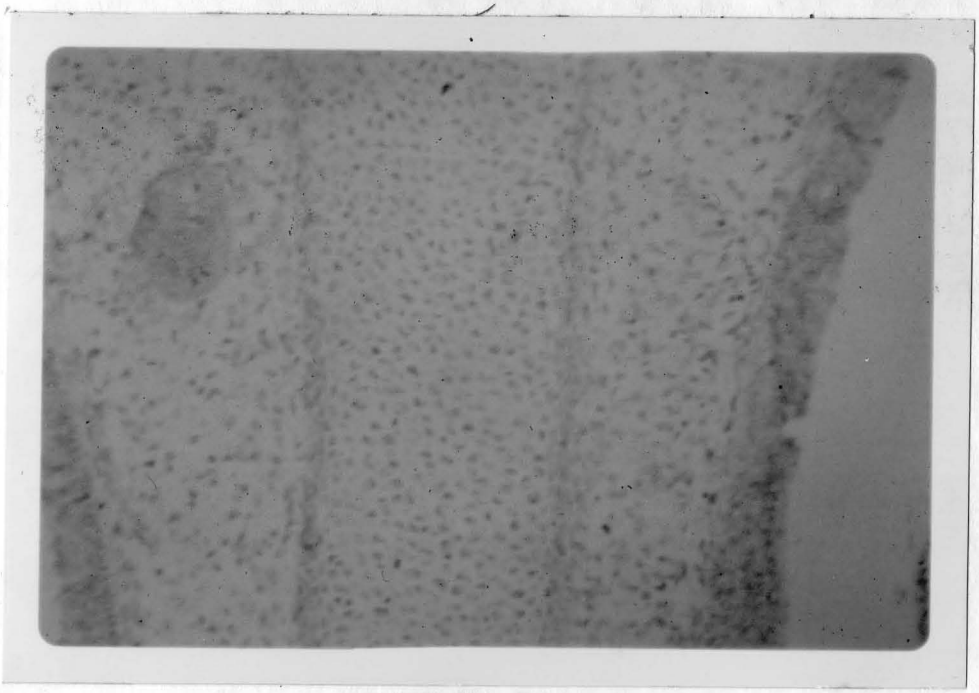

FIGURE 12

## PLATE VII

## EXPLANATION OF FIGURES

13 Tissue section of nasal cartilage incubated in 50 ml of distilled water pH 5.5 containing PTE for 48 hours and stained with methylene blue. Tissue was fresh frozen. Note lack of discernible walls on the lacunae, compare to figure 14. X100.

14 Tissue section of Meckel's cartilage incubated in 50 ml of distilled water pH 5.5 containing PTE for 48 hours and stained with methylene blue. Tissue was fixed in F.A.A. The photomicrograph is representative of the residual metachromasia after PTE treatment described in the text when formaldehyde fixatives were used. It is to be contrasted to figure 11 where almost no residual metachromasia was observed in tissue fixed in non-formaldehyde containing fixatives. X100.

FIGURE 13

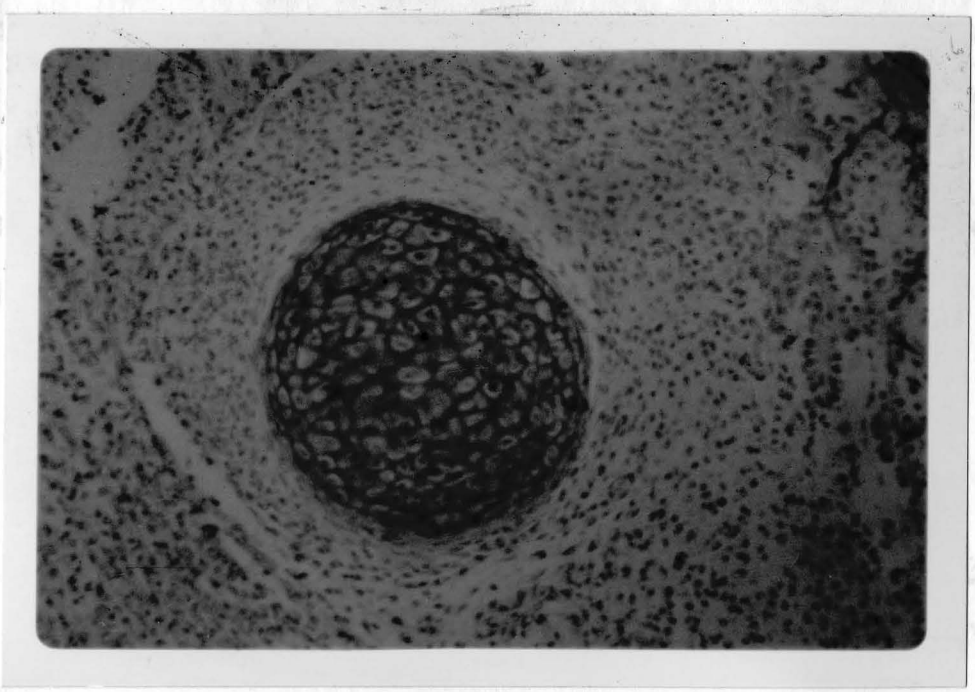

FIGURE 14

APPROVAL SHEET

The thesis submitted by Nicholas Joseph Malinski, Jr. has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 22, 1968
Date

Robert C Clawson
Signature of Advisor