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**MUCOPOLYSACCHARIDES IN THE ORAL MUCOSA
OF THE THYROIDECTOMIZED RAT**

by

Rodrigo Eisenmann Grimas

**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**

June

1962

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LIFE

Rodrigo Eisenmann Grimas was born on September 30, 1932, in David, Republic of Panama.

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TABLE OF CONTENTS

<u>Chapter</u>		<u>Page</u>
I.	INTRODUCTION	1.
II.	REVIEW OF THE LITERATURE	2.
	A. <i>Connective Tissue</i>	2.
	B. <i>Thyroid</i>	21.
	C. <i>Thyroid Influences in Connective Tissue</i>	24.
III.	MATERIAL AND METHODS	29.
IV.	FINDINGS	33.
V.	DISCUSSION	41.
VI.	SUMMARY AND CONCLUSIONS	51;
VII.	BIBLIOGRAPHY	53.
VIII.	APPENDIX	60;

INTRODUCTION

The different hormonal effects on the connective tissue have been subject to intense investigation. In the case of a deficiency of circulating thyroid hormones--hypothyroidism, it has been seen that changes in the loose connective tissue of skin does occur bringing the appearance of the pathological entity called myxedema. The skin of myxedematous patients has been studied by histochemical means, and it has been reported that the main disturbance is an abnormal accumulation of acid mucopolysaccharides in the ground substance of the connective tissue.

It has been reported that thyroidectomy causes the appearance of myxedema in the skin of rats.

The loose connective tissue of the oral mucosa and of the skin are similar, and it could be expected that hypothyroidism would cause a similar alteration in both sites. However, the reports found in the dental literature about this condition are very limited.

The purpose of this experiment is to study the oral mucosa of thyroidectomized rats with special attention to the mucopolysaccharide components of the connective tissue.

REVIEW OF THE LITERATURE

A. Connective Tissue

Connective tissue is widely distributed in the body. Despite the fact that there are different kinds of connective tissue, with more or less special characteristics, all of them have certain common features. Thus, the basic structural components are always cells, fibers, and the "ground substance".

Ground Substance: *The physicochemical state of the ground substance has been intensely studied. Electronmicroscopy, biochemical assays, histochemical and electrochemical methods have been used.*

It has been stated (24, 25) that the ground substance is a heterogeneous colloid, largely formed of macromolecules of carbohydrate-protein complexes (mucoproteins and glycoproteins) and mucopolysaccharides, that give at physiologic pH, an electronegative charge. Thus the tissue exhibits the behavior of an immobile charged colloidal polyelectrolyte, whose composition varies from tissue to tissue. Loose connective tissue, yields a low negative charge, while very dense connective tissue such as bone or dentin shows a greater density of negatively charged colloid. Because of the negative charge, the biological colloid gains unique properties affecting the water and salt distribution and the organic and inorganic cation bindings in the tissues (24). The role of connective tissue colloidal components in

influencing the diffusion and exchanges of electrolytes and other solutes is very important. It is now recognized that they are not just an inert mass through which electrolytes and metabolites pass from the circulation to the cells (72). The distribution of diffusible ions is determined primarily by the indiffusible charged macromolecules of acid mucopolysaccharides and carbohydrate-protein complexes (29).

According to Gersh and Catchpole (28,29), the components of the ground substance are as follows:

Table I.

		Hyaluronic acid
		Chondroitin sulfate A
		Chondroitin sulfate B
	Acid mucopolysaccharides	Chondroitin sulfate C
		Chondroitin
		Kerato sulfate
		Heparitin sulfate
	Neutral heteropolysaccharides	
	Protein (s)	
	Soluble collagen	
Probably of local origin	Water	
	Enzymes	
	Metabolites	
	Immune bodies	
	Albumin	
	Globulin	Originating from plasma
	Vitamins	
	Hormones	
	Ions	

The ground substance consists of water soluble and water insoluble parts, which makes it a two-phase system: colloid-rich water-poor and water-rich colloid-poor (28, 29). Bondareff (18) studies the ground substance with the aid of the electromicroscope and described a system of vacuoles, with dimensions ranging from 500A to 2,000A, inclosed in a dense continuum. The walls of the vacuoles are believed to be rich especially in proteins, while the chief constituent within the vacuoles is water. Gersh and Catchpole (29) stated that the components derived from plasma, in addition to those of local origin are incorporated in this two-phase system. There is an equilibrium state between the submicroscopic vacuoles and their walls, which in turn are in equilibrium with the blood plasma.

The amount of water of the connective tissue depends on the intrinsic hydrophilic properties of the colloid, colloidal charge density, blood pressure, osmotic pressure of the blood plasma, lymphatic drainage, etc. (29). With regard to the hydrophilic properties of the colloid, Slack (72) considered that the water in connective tissue is bound to the mucopolysaccharide-mucoprotein-fibrous protein complexes. That, when connective tissue swells, there is an increase hydration of the ground substance and collagen fibers; the hydration of the ground substance preceeding the swelling of the collagen fibers. These changes in water binding seem to be initiated by alterations in the ratio of free and colloid-bound electrolytes

of the tissue. It has been demonstrated (41) that one of the components of the ground substance, hyaluronic acid, is strongly hydrophilic and has a definite role in the water binding capacity of the tissue. The greater the amount of hyaluronic acid, the richer in water the tissue is. Gersh and Catchpole (29) also report that the state of aggregation or polymerization of the ground substance is of primary importance for the accumulation of water in the tissues. The disaggregated ground substance is colloid-poor but rich in water; while the more highly aggregated ground substance is colloid-rich and water-poor.

Gersh and Catchpole (29) stated that the properties and reactivities of the ground substance are the following:

a) It constitutes the actual environment of most cells of the organism. It is the "milieu interieur" through which nutrients, metabolites, ions and water pass. The binding capacity of the ground substance makes it the great reservoir of water and ions and determines their relative concentrations in the cellular environment.

b) It is an important means of stabilizing the spatial relations of cells and tissues in organs and of maintaining them in functional relations.

c) It is the "mother liquor" from which are extracted the components of collagen and reticular fibers and the denser organic components of cartilage, dentine and bone.

d) *It is critically important in protecting the organism against bacterial invasion and certain forms of trauma.*

e) *It plays an important part in the changes developed during growth and differentiation, regeneration, and aging.*

f) *It responds to hormones.*

g) *Its nature is altered in various pathological conditions: inflammation, rickets, periodontosis, scurvy, arteriosclerosis, and at least some of the collagen diseases.*

The mucopolysaccharide fraction of the ground substance (Table I.) has a great biological importance. Their essential components are hexosamine and hexuronic acid; derivatives of either glucose or galactose. Glucosamine is a stable amino sugar derived from the replacement of a secondary hydroxyl (in the two-carbon position) by an amino group; while glucuronic acid is a monobasic acid, with an aldehyde remaining at the opposite end of the straight carbon chain from the carboxyl group (70).

K. Meyer (54) suggests this classification for the mucopolysaccharides.

Neutral: *containing no acid groups (e.g. chitin: composed of acetylglucosamine only)*

Acid: a) **Simple:** acid component, uronic acid (e.g. hyaluronic acid: composed of acetylglucosamine and glucuronic acid) and

b) **Complex:** acid component, uronic acid and sulfuric or phosphoric acid (e.g. corneal mucoid, heparin: composed of glucosamine or acetylglucosamine, uronic acid and sulfuric acid; chondroitin sulfuric acid: composed of acetylgalactosamine, glucuronic acid and sulfuric acid).

Hyaluronic acid: In 1934, Meyer and Palmer (57) isolated from bovine vitreous humor, a high molecular weight polymer of a disaccharide, which consisted of equimolecular acetylglucosamine and glucuronic acid. They named it "Hyaluronic acid". It was later also isolated from umbilical cord (58), human skin (62), oral mucosa (71), etc. Pearce and Watson (62) have found hyaluronic and chondroitin sulfate to be present in human skin in approximately equal amounts. However, Meyer and Chaffee (56) isolated 1.08 gm. of chondroitin sulfate from the total mucopolysaccharide content of 1.85 gm. extracted from 2.28 kg. of fresh hog skin. Meyer found that hyaluronic acid was the only mucopolysaccharide present in synovial fluid and vitreous humor, while it is absent in the cornea and hyaline cartilage.

Stacey (73) gives this approximate values for the components of hyaluronic acid, extracted from different sources:

Nitrogen	3- 3.5	%
Glucosamine	30-40	%
Glucuronic acid	40-50	%
Acetyl	8-12	%

From data on viscosity and streaming birefringence of flow, Blix and Snellman (14) estimated a mean particle length for purified neutral hyaluronate of between 4,700 and 10,000 Å. The highly asymmetric chain is thought to be unbranched. Hyaluronic acid is polydisperse in nature, and its particle size and weight depend upon the source and method of extraction, but a suggested molecular weight was between 200,000 and 500,000 assuming a disaccharide length of 10 Å. (70). Rothman (70) states that purified hyaluronic acid also has these qualities----- is extremely hygroscopic: is not antigenic: and does not have x-ray diffraction and crystalline structure. Hyaluronic acid has no regular fiber structure under the electron microscope (33).

Meyer (50) considers that hyaluronic acid forms reversible polar complexes with protein but is not chemically bound to it; that at the physiologic pH of tissues, hyaluronic acid is free of protein. Blix (13) and Ropes et al (67) have supported this conception by electrophoretic studies of synovial fluid from cattle. Synovial fluid at pH 7.6 in an electrical field has a fast moving polysaccharide component which is separate from the two slower moving albumin and globulin protein components. Ropes et al (67) compared the native synovial fluid with fluid that had been treated with trypsin in order to destroy the protein and liberate the hyaluronic acid. They found no difference between the viscosity of the

joint fluid before and after proteolysis and ascribed the viscosity entirely to hyaluronic acid. Moreover, further addition of protein made no change in the viscosity.

However, Ogston and Stakier (61) used ultra-filtration as a method of isolating the hyaluronic acid-protein complexes from the joint fluid of cattle. They found that the fraction containing hyaluronic acid was all within a sharply defined filtration separation and that all such samples contained protein. Any attempt to separate the protein caused degradation of the hyaluronic acid, as judged by viscosity and ultracentrifugation. Thus, we can see that the relation of hyaluronic acid to the proteins of the ground substance is not clear.

Chondroitin sulfate: Chondroitin sulfuric acid has been found in a wide variety of connective tissues. Meyer and Rapport (59) found that cartilage, bone, cornea, aorta, and the ligamentum nuchae contained chondroitin sulfate A. Chondroitin sulfate C occurs in cartilage and also in umbilical cord, tendon and nucleus pulposus. Chondroitin sulfate B has been isolated from skin, tendon, heart aortic valves, and ligamentum nuchae.

Chondroitin sulfate on hydrolysis yields sulfuric acid, acetic acid, glucuronic acid and chondrosamine (2-desoxy-2 aminogalactose), combined in equimolecular proportions (70). Blix and Snellman (14) estimated that the mean particle length was about 4,700 A. with a molecular weight of the

order of 260,000, from data on streaming birefringence. However, Mathews and Dorfman (50) extracted a chondroitin sulfate of high purity for a study of its physicochemical properties, and this material gave a molecular weight of 43,000. It must be considered that the chondroitin sulfate is firmly bound to proteins and is very difficult to extract without altering its chemical and physical properties.

Meyer and Rapport (59) found that chondroitin sulfates from different tissues differ in specific optical rotation, solubility and resistance to enzyme hydrolysis. Thus chondroitin sulfate A from hyaline cartilage is different from the type B from skin and the C from umbilical cord and tendon. Chondroitin sulfate B has an optical rotation of -60 degrees; its calcium salts are less soluble than either A or C, and it is completely resistant to hydrolysis by testicular or bacterial hyaluronidase. Pearse (63) reports that the Chondroitin sulfate A is labile to testicular and pneumococcal hyaluronidase, while the chondroitin sulfate C is labile to the testicular but not to the pneumococcal hyaluronidase.

Chondroitin sulfate is very much less viscous than hyaluronic acid and is thought not to play a significant role in the water binding of the connective tissue. The highly charged anionic groups of chondroitin sulfuric acid probably play a more important role in the binding or transfer of electrolytes (70).

Chondroitin sulfate is firmly bound to nonfibrous proteins. Meyer (55) suggests that the chondroitin sulfate-protein complex contain about 35 to 40 per cent of carbohydrate. The protein is distinct from collagen and is easily digested by trypsin, with a relative high yield of tyrosine and tryptophane.

Chondroitin: *This non-sulphated polysaccharide appears to be an isomer of hyaluronic acid in which the D-glucosamine is replaced by the D-galactosamine. Like hyaluronic acid, it forms viscous solutions, gives a mucin clot on acidification and is digested by testicular and bacterial hyaluronidase. It has been isolated only from cornea (72).*

Keratosulfate: *This is a sulfated mucopolysaccharide which is free of uronic acid. It is composed of equimolar amounts of N-acetylglucosamine, galactose and sulfate. It has been isolated from cornea and nucleus pulposus (72).*

Heparitin sulfate: *This mucopolysaccharide fraction has been isolated from bovine aorta and from human amyloid tissue. It is composed of equimolar amounts of glucosamine uronic acid and sulfate and is resistant to testicular hyaluronidase (72).*

Little is known of the chemical synthesis of the mucopolysaccharides. Mosbach and King (60) fed C^{14} labeled glucose to guinea pigs and found that the carbon chain of glucose is directly available for conversion into

glucuronic acid. Roseman et al (69) nurtured a strain a group A hemolytic streptococcus which gives a high yield of hyaluronic acid, in a medium in which was incorporated glucose labeled with a radioactive carbon. It was found that glucose was the main source of carbon for hyaluronic acid and also that the 1-carbon labeling was detectable in the glucosamine yield on hydrolysis. Nothing is known of the mechanism of polymerization of the mucopolysaccharide complexes, and there is no evidence that hyaluronidase plays any part in synthesis. There may, however, be enzymatic control of polymerization as this has been observed to take place in other biological systems, even extra-cellularly, either by addition or condensation (70).

Histochemistry of the ground substance: The histochemical determination of mucopolysaccharides is based on their metachromatic characteristics. When these substances are stained with certain single-colored cationic dyes, they are capable of forming an entirely different color. Ehrlich in 1879 was the first to call this phenomenon 'metachromasia', the change in "colour". Among the dyes methyl violet, thionine, the azures and toluidine blue are the more commonly used.

Sylvén (74) and Pearse (63) state that the reaction signifies only the presence of free electronegative surface charges of a certain minimum density. It concerns many polyelectrolytes and is not indicative of any specific chemical compound.

The substances (chromotropes) which produce metachromasia in animal tissues are large macromolecular colloids, with spaced acidic groups, i. e. $-\text{OSO}_2\text{OH}$, $-\text{COOH}$ and perhaps $-\text{OPO}(\text{OH})_2$ to which the basic dye is fixed (63). Sylven (74) considered the following factors as essential for the appearance of metachromasia: a) An orderly alignment of the attached dye molecules. This is favored if the dye molecules have hydrophobic and hydrophilic parts. Metachromasia involves two principal steps: first the interaction between the dye and the substrate molecule and, secondly, interaction between adjacent dye molecules aggregated to the substrate. Sylven believes that a molecule of water might be intercalated between the dye molecules, thus giving a hydrogen bond between them. The presence of a hydrophilic part in the dye molecule, in a constant relation with the hydrophobic part, will favor the orderly interaction of the dye molecules. b) The distribution of electro-negative surface charges of the substrate determines the attaching alignment of the dye molecules and the degree of metachromasia. A certain minimum interchange distance is a requisite for the reaction. The minimum distance is estimated about 5 Å. The degree of metachromasia will increase when a higher aggregation of the attached dye molecules is present, bringing the interchange distance to less than 5 Å. Stain is favored by high dye concentration and alkaline pH (increasing hydration). c) The charge quality of the

substrate also is important in the reaction. Thus, a sulphate radical will give an intenser reaction than a carboxyl group: d) The presence of water is essential for metachromatic interaction: e) A certain threshold of dye is apparently necessary before metachromasia occur. Sub-threshold amounts of dye, in the case of the heparin for example, will produce orthochromatic aggregates. This quantitative dye-substrate ratio is important also because it has been observed that an excessive amount of dye, will give a metachromatic substance, and orthochromatic appearance.

Sylvén (74) considered that the spectral absorption shift, or metachromasia, is due to the new bonds appearing between adjacent dye molecules.

Rothman (70) considered that the substances ordinarily showing metachromasia are large molecules of sulfate esters, such as mast cell heparin, chondroitin sulfates, and mucosin sulfuric acid. Sylvén (14) demonstrated that hyaluronic acid, in order to show metachromasia, needs to be highly polymerized and in a minimum concentration of 1 per cent (w/vol.). In lower concentration, it gives an orthochromatic reaction.

The histochemistry of polysaccharides is closely linked to the periodic-acid Schiff reaction (PAS). Its use in histology was first described by McManus (52) for the demonstration of mucin, but later Lillie (47) and Hotchkiss (40) elaborated the method into a histochemical one which could be used for the detection of different polysaccharides in the tissues.

Periodic acid (HIO_4) is an oxidant, which breaks the carbon bonds (C-C) where these are present as 1:2-glycol groups (CHOH-CHOH), converting them into dialdehydes ($\text{CHO}.\text{CHO}$). The equivalent amino or alkylamino derivatives of 1:2 glycol or its oxidation product ($\text{CHOH}.\text{CO}$) are also attacked and converted into dialdehydes. The particular property of periodic acid is that it does not further oxidize the resulting aldehydes, and these, then, can be localized by combination with the Schiff's reagent to give a substitute dye which is red in color (63).

According to Hotchkiss (40), positive results should be obtained when the treated substances fulfill the following requirements: a) Contains the 1:2 glycol grouping or the equivalent amino or alkylamino derivative, or the oxidation product $\text{CHOH}.\text{CO}$. b) Do not diffuse away in the course of tissue fixation. c) Give an oxidation product which is not diffusible. d) Are present in sufficient concentration to give a detectable color.

Pearse (63), Hotchkiss (40), and other authors believe that the naturally occurring animal substances that give a PAS positive reaction are the monosaccharide, polysaccharide, neutral mucopolysaccharides, mucoproteins, glycoproteins, glycolipids, unsaturated lipids and phospholipids. Glegg et al (30) found that these complexes contained as their main carbohydrate components, the hexose sugars glucose, mannose and galactose; together with the methylpentose sugar fucose and various

hexosamines. All the mentioned authors agreed in the fact that acid mucopolysaccharides are not PAS positive. Also Braden (15) and Hoogwinkel and Smits (39), carried in vitro tests to demonstrate the PAS reaction of acid mucopolysaccharides. They found that chondroitin sulfuric acid and hyaluronic acid, both were PAS negative. They noted that although the hexuronic acids were oxidized, no aldehydes formation could be demonstrated.

There is a diffuse PAS staining in the connective tissues. Particular condensations are seen at the junctions of connective tissue with epithelium and outside the endothelium of the capillaries (Basement membranes).

The treatment of the tissues with various enzymes will bring modifications in the results of the previously mentioned stainings. They could either act by removing the reactive groups by hydrolysis, such as in the case of hyaluronidase and amylase with the consequent reduction in metachromasia and PAS or uncovering reactive groups by breaking polysaccharide-protein complexes such as in the case of proteolytic enzymes in which the more available carbohydrates will give a more intense result with PAS.

The methods of fixation are of critical importance. The fixative could combine with some of the components of the tissue blocking the expected reaction. Also the presence of water soluble components in the

ground substance must be considered in the case where a fixative in aqueous solution is used. In this case, the water soluble components will be washed out with the subsequent decrease in stainability of the tissue. Also, there could be a diffusion of the components of the ground substance to areas where they would not be present normally. The method of freeze-drying avoids many of these complications. It respects more the general characteristics of the tissue and permits a better evaluation of the quantity and quality of the tissue components. This method of fixation consists of a rapid "quenching" of the tissue in temperatures of -160°C ., with a subsequent dehydration under temperatures of -60°C . (63).

Fibers: *The connective tissue contains three kinds of fibers, collagen, reticulin and elastin that belongs to the group of fibrous proteins made up of elongated chains of amino acids joined together by the peptide linkage, the chains themselves being held in more or less parallel alignment by various cross-linkages (70).*

Collagen is the major component of the connective tissue. It is perhaps the most abundant protein in the animal kingdom. It has a relative high content of glycine and proline and accounts for practically all the hydroxyproline found in the body. This amino acid and also hydroxylysine seem to be formed after the molecule chain has been assembled; the new amino acids are created by addition of hydroxyl groups to some of the proline and lysine

units in the chain. This alteration of the primary molecular structure has not been observed in the synthesis of other proteins. Proline and hydroxyproline impart rigidity and stability to the collagen molecule (35). Under the electronmicroscope, collagen shows a periodicity of 640 Å (35, 43, 47).

Collagen is synthesized by the fibroblast. Actually, it is still not very clear whether these cells elaborate collagen fibers as such or a precursor. It seems more probable that they elaborate a precursor that is deposited extracellularly and from which, by aggregation, the actual fiber is formed (44). An alkali soluble, a citrate soluble and an insoluble fraction of collagen have been discovered. Jackson (44) states that the alkali soluble fraction is the real precursor of the fiber and is the one that is synthesized intra-cellularly. It has been shown (44) that it is the fraction that has a higher metabolic incorporation of C^{14} labeled glycine. The next aggregated fraction is the citrate soluble which shows a lower C^{14} labeled glycine metabolism. Then, a series of cross-linkages, presumably in the H bonding between the OH groups of hydroxyproline and the keto-imide groups of adjacent helices (36), makes it a mature insoluble collagen fiber.

Rothman (70) states that pepsin digest collagen, while the action of trypsin is not very clear. Seemingly, it produces, with difficulty, only mild changes in the collagen molecule.

It has been thought that mucopolysaccharides are necessary agents

for the formation of collagen fibers. Gross (34) shows the precipitation of collagen fibers, in vitro, by chondroitin sulfate. Also it must be noted the fact of increase metachromasia before any visible fiber can be observed in the case of wound healing. However, Wolbaach (80,81) has demonstrated the formation in vitro of collagen fibers starting from the neutral solution of collagen and in the absence of mucopolysaccharides. We can see, therefore, that the role of mucopolysaccharides in fibrinogenesis is still obscure.

Reticulin fibers characteristically stain black with the silver impregnation method. It is known (45) that the basement membrane type of reticulin is composed of collagen fibers in very firm association with a carbohydrate and a lipid component. Only after hydrolysis is there a separation of these components (48, 79). It has also been shown (21) that antiglomerulus serum rendered fluorescent by the technique of Coons and Kaplan, stained the basement membrane fibers but not the collagen fibers, indicating that an antigen not present in collagen is a component of reticulin. Robb-Smith (65) found that the extraction of thin slices of human kidney with a variety of solutions (dilute acetic acid, sodium hydroxide) had a marked effect on the staining qualities of the collagen fibers and reticulin fibers of granulation tissue, but none on the reticulin fibers of the basement membrane. Therefore, the presence of two types of

reticulin fibers has been assumed, one in granulation tissues and the other, the more "mature", in the basement membrane (s) (44).

Elastic fibers are scarce in the loose connective tissue. They appear as brilliant, highly refractive, cylindrical threads, much thinner than the collagenous fibers (51). Rothman (70) states that x-ray diffraction analysis of unstretched elastic fibers gives an amorphous pattern, in contrast with the oriented, fibrillar type, that gives collagen. An albuminoid, elastin, is the characteristic constituent of these fibers. It is highly resistant to boiling water, acids and alkalies. Elastin is slowly digested by trypsin, pepsin, and elastase (51).

Cells: *The cells usually found in loose connective tissue are the: a) Fibroblasts which are the cells that enter into the synthesis and organization of the different fibers and ground substance. b) Undifferentiated mesenchymal cells; these are the cells usually arranged along the blood vessels, particularly capillaries and which some authors believe (51, 62) have the pluripotential capacity of becoming a new differentiated cell type such as an osteoblast or a fibroblast under the influences of certain stimuli. Mast cells, which are also found often in groups about the blood vessels.*

Their cytoplasm is filled with granules that are strongly metachromatic when stained with basic aniline dyes. The presence of histamine and a heparin-like substance in their cytoplasm has been demonstrated (70).

Their function as producers of ground substance, especially the acid mucopolysaccharide fractions, is the subject of intense investigation.

Asboe-Hansen (3, 9, 10) have postulated that they will respond to hormonal influences, either by increasing in number and in granulation, as in the case of TSH stimulation, or by losing their granules and decreasing in number, as in the case of cortisone.

Also present in the loose connective tissue are macrophages and a very small number of lymphoid wandering cells eosinophils, plasma cells, pigment cells and fat cells (51).

B. Thyroid

The secretions of the endocrine glands, the hormones, constitute a system for regulating the rates of growth, development and function of certain tissues and the rate of many metabolic processes within the body (26). All the glands of this complicated system work in a very close interdependence. They are subject to control by the Central Nervous System, by other endocrine glands, by certain metabolites or by a combination of these factors (51).

In this way, we can understand of the existence of the "Pituitary-Thyroid Axis". The secretion of Thyroid Stimulating Hormone (TSH) from the anterior lobe of the hypophysis causes an increase in the synthesis and release of thyroid hormones, and stimulates the growth of the thyroid

gland. In the same way, an increase of circulating thyroid hormones in the blood, will "feed back", and reduce the release of TSH from the hypophysis. A diminution will bring about a greater release of TSH (17, 26, 68).

Stimulation of the paraventricular nuclei of the hypothalamus, has produced an increase in the amount of secretion of TSH (38). However, it is not clearly known, if the influence of the blood level of thyroid hormones has a direct action on the hypothalamus (20). By way of neuro-secretions carried through their "Portal system", the hypothalamus will, in turn, stimulate the hypophysis causing the release of thyrotrophic hormones (26).

The Central Nervous System acts indirectly by way of the hypothalamus. It is known (28, 56) for instance, that changes of temperature, stress conditions, etc., will bring alterations in the production and release of thyroid hormones.

The thyroid gland, develops from an epithelial budding on the floor of the primary oral cavity (64). It lies, with its attendant para-thyroid, on the lateral surface of the trachea, just below the larynx. In the rat, it covers 4 to 5 tracheal rings. The two thyroid lobes are joined by an isthmus. The para-thyroid is closely applied to the antero-lateral surface of the thyroid (32). Each anatomical lobe of the thyroid is composed of a number of smaller structural lobes. Each structural lobe consists of many lobules. These are made of a number of follicles bound together by

fine connective tissue. The follicles are filled with a colloidal substance, mainly composed of thyroglobulin, iodothyroglobulin, mucoproteins and enzymes (27).

According to Pitt-Rivers (64), in the normal thyroid gland, synthesis of the hormone occurs in these stages: a) concentration of iodide from the blood, b) iodination of Tyrosine yielding mono and diiodotyrosine, c) conversion of these to Thyroxine and Triiodothyronine. Then, these iodoamino acids are stored in the proteinthyroglobulin. Later, after the hydrolytic action of thyroid protease, thyroxine and Triiodothyronine are secreted into the blood stream. Here again, these iodoamino acids will be carried through the blood to the "target" tissues or cells. Circulating thyroid hormones reach the extracellular space of different organs according to their nature before they are picked up by the cell receptors. The efficiency of this last process varies in different organ, the liver being especially active (66).

In order to discover the "active" form of the thyroid hormone, their metabolic pathways have been intensely studied (66). However, it is still not clear which metabolite is the acting end-product.

The mode of action of the hormone is not clear either. It is known that its presence brings an increase in the cellular metabolism, but the exact nature of its role has not been discovered.

C. Thyroid Influence in Connective Tissue

As early as in the 1890's, thyroid extract began to be used in the treatment of hypothyroidism. And it was readily observed that the administration of this extract brought an immediate resolution of the clinical symptom, so-called "myxedema". This term was used to describe the abnormal accumulation of mucinous substances in the dermal connective tissue. This was viewed as an example of a direct inter-relation of the connective tissue and a hormonal action.

Byron (19) measured the fluid and electrolyte loss in subjects receiving thyroid extract. In the normal, there is more potassium than sodium excreted indicating loss of intra-cellular fluid; while in the myxedematous subjects, an excess in sodium is evidenced that the accumulation of fluid is mainly extra-cellular. Brewer (16) in an autopsy study of hypothyroid subjects, reported the gross accumulation in the skin of metachromatic acid mucopolysaccharides. Asboe-Hansen (2, 3) and Asboe-Hansen and Iversen (7, 8) stated that the increased acid mucopolysaccharide fraction in myxedema, as well as pretibial myxedema and exophthalmus, was hyaluronic acid. They deprived hypothyroid patients of thyroid extracts, in order to make them myxedematous and found an accumulation of metachromatic material and of Ehrlich Mast cells. This metachromatic material was sensitive to testicular and bacterial hyaluronidase.

They concluded that the accumulation of water was due to the water binding capacity of the hyaluronic acid. However, Watson and Pearce (77) carried out alkaline extractions of the interfibrillar mucinous material, of large specimens of pretibial myxedema. They compared it with normals from amputated legs and reported an increase of both hyaluronic acid and chondroitin sulfate:

	Water Content (%)	Free acid (Mg. per 100 Gm. fresh skin)	
		Hyaluronic a.	Chondroitin s.
Normal skin	61.0	24.5	26.2
Pretibial myxedema	74.8	63.6	48.7

Ropes (67) also reported that the viscosity of the joint fluid is increased in myxedema.

Asboe-Hansen (5, 6) reports in pretibial myxedema, which he states can be present in hyperthyroidism as well as in hypothyroidism, a thickening of the skin; and in connective tissue, the accumulation of hyaluronate and chondroitin sulfate. He described signs of degeneration in the collagenous fibers as "bursting" apart, because of the mucinous edema. They also described the presence of Mast cells in the perivascular areas and other parts of the connective tissue. He proposed that in these cases of localized myxedema, there might be an increased capillary permeability and a tendency to edema along with the increased mucopolysaccharide formation.

In generalized myxedema, he described similar changes, but less marked. Also, the sub-epidermal tissue exhibits increased metachromasia, in contrast with the circumscribed myxedema in which this layer is almost normal, and the mucinous edema is only present in the deeper layers of the corium.

Asboe-Hansen (7) believes that the increased secretion of TSH is the necessary stimulus that will bring an elevation in the number of Mast cells and the liberation of their granules, thus causing as an end result, the greater amount of hyaluronic acid. He injected excessive amounts of TSH in thyroidectomized (TX) guinea pigs and reported the appearance of exophthalmus. Ludwig et al (49) injected TSH in normal and TX guinea pigs and found an increase in the amount of water and glucosamine-containing ground substance in the TX guinea pigs injected with TSH; while the dry weight of the tissues both normal and TX remained the same. Dodds and Robertson (23) used dinitro-orthocresol in order to increase the metabolic rate of myxedematous tissues. However, in this case, even though there was an increase in the metabolism, there was no reduction of the accumulated mucin in the ground substance. This was used by Asboe-Hansen as further evidence that the actual action of the thyroid hormones in the reduction of myxedema is by blocking the secretion of TSH from the pituitary and not a direct local one in the peripheral tissues.

Del Conte et al (22) report biochemical assays and histochemical examinations of skin of TX rats, and their results show an increase concentration of the dermal acid mucopolysaccharide and also of Mast cells.

Baume and Becks (11) worked with rats thyroidectomized at birth, and among their findings, report a decrease in the cellular and vascular components in the lamina propia of the oral mucosa. This they believed made the fibrous components more prominent. They reported that the greater changes were in the epithelial structures, where the proliferation of germinal cells was slow, histodifferentiation retarded, and the life cycle of mature epithelial cells shortened. These facts, they believed, were responsible for the scaly skin and oral mucosa of hypothyroid rats.

However, Ziskin and Stein (82) using TX rhesus monkeys, described degenerative changes in the connective tissue of the oral mucosa, as well as in the epithelium. They found increased mitosis, hyperkeratosis, intercellular edema, and pyknotic nuclei in the epithelium. In the connective tissue, they found an increase in the cellular and vascular elements, coarsening and thickening of the fibers with edema between them. They also mentioned, that "special stains" disclosed an increased amount of mucous staining material and glycogen.

The occurrence of a pathological state, such as myxedema, in the oral mucosa, of of importance. Whether its loose, connective tissue is

affected in hypothyroid conditions has not been clearly studied.

The desire to evaluate, with histochemical means, this possible alteration has prompted this investigation.

MATERIAL AND METHODS

Thirteen male albino rats were used. Nine of these animals were thyro-parathyroidectomized by the commercial breeder¹ in such a way that the Thyroid and Parathyroids were removed in toto at the age of four weeks. The animals were received in our laboratories a month later. The animals were fed regular Rockland Rat diet. They were sacrificed at the age of six to seven months by ether and then decapitated. The four control animals of the same strain were of approximately the same age. The average weight of the TX animals at the time of the sacrifice was 212 gm., while the normal "control" animals had an average weight of 269 gm. Besides this difference of weight, no other visible physical difference was observed between TX and normal animals. The necks of the TX animals were preserved in 10% formalin and later a gross dissection was done in order to expose any possible remnants of thyroid gland. No thyroid tissue was found.

Specimens of tissue were obtained from the oral mucosa of the cheek and vestibule and from the attached gingiva of the molar and incisor region. Also a sample of skin from the ventral surface of normal and TX rats was secured.

1. The Charles River Breeding Lab.

The majority of the specimens were treated by freeze-drying technique. First, they were submerged in iso-pentane, pre-chilled with liquid nitrogen to -160 C., and later dehydrated for a period of four days at a temperature of -60 C. Other of the specimens were fixed for 48 hours in an acridine-ethanol solution, as recommended by Pearse (63) as a good method for the fixation and immobilization of acid mycopolysaccharides. A number of the normal and TX specimens were fixed for 48 hours in an alcohol-acetic acid solution in order to obtain sections for a better morphological study of the tissue.

The embedding was done in vacuo with Paraffin (Tissuemat Fisher Company) of a melting point of 56.5 C., under 15 pounds of pressure over a period of 15 minutes.

Sections were cut from 6 to 10 microns. The thickest sections were used in enzyme incubation procedures, while the others were used to observe clearly the cell definition.

One group of the mounted slides were deparaffinized with petroleum ether and then post-fixed in absolute alcohol overnight. Another series was deparaffinized in the following manner: Immersion for five minutes each in two changes of Xylol; then five minutes in 100% alcohol, three minutes in 95% alcohol and three minutes in 75% alcohol. Finally, the sections were washed in distilled water for five to ten minutes. All the tissues fixed in

alcohol-acetic acid solution and acridine-ethanol solution were deparaffinized in the second manner.

Two methods were employed for the demonstration of acid mucopolysaccharides. The first was Toluidine Blue at a concentration of 1:1000 and a pH of 7 (53), and the second was Thionin at a concentration of 5:100 and a pH of 7 (37). A number of sections thus prepared were mounted in water and examined immediately while others were mounted in Permount to preserve them for subsequent examination.

Testicular Hyaluronidase Incubation (Wyeth and Nutritional Biochemicals) was used for the removal of hyaluronic acid, chondroitin sulfate A and C. The following procedure was used: A solution of a concentration of 1 mg. of testicular hyaluronidase (approximately 150 TRU) per 1cc. of Sorensen's Phosphate buffer at a pH of 7 was made. Five drops of the above solution were used to cover each deparaffinized slide. The slides were placed in a Petri dish containing a wet chamber and then incubated at 37 C. for three hours. Control slides were treated with Sorensen's buffer solution alone and then incubated at the same temperature and for the same length of time. Immediately following incubation, the sections of both the normal and experimental animals were stained by either the toluidine blue or thionin methods.

For the demonstration of neutral polysaccharides the Periodic-acid

Schiff (McManus modification of Coleman) method was used. Light green, a relatively non-specific plasmas stain was used as a counter stain (53).

Incubation in amylase for the removal of glycogen was also used in the following manner: A solution of a concentration of 1mlg. of amylase (Nutritional Biochemicals) per 1cc. of Sorensen's Phosphate buffer at a pH of six was made. Five drops of the solution were used to cover each deparaffinized slide: the slides were placed in a Petri dish containing a wet chamber and then incubated for a period of 30 minutes at 37 C of temperature. Control slides were treated with buffer solution alone and incubated at 37 C for 30 minutes. Immediately following incubation, the sections were stained with the periodic-acid Schiff method.

In addition, sections were stained with Gomori silver impregnation and Heidenhain's Azan stain (modification of Mallory's connective tissue stain) to study the fibers components of the connective tissue. The first one to differentiate the argyrophilic reticulin fibers from the rose-colored mature collagen fibers; and the second method to have a general picture of the total content of the similarly blue stained collagen and reticulin fibers.

To study the general morphology of the tissues, sections were stained with the haematoxylin and eosin method.

FINDINGS

Toluidine Blue: No significant difference was observed in the epithelium of both normal and TX animals, regardless of the site from which the specimen was obtained. The nuclei of all the epithelial cells stained metachromatically, with the nuclei of the basal cell layer giving the most intense reaction. The cytoplasm of the cells of the germinal layer; that is the basal and lower spinous layer, usually gave an orthochromatic reaction, while in the upper spinous and granular layer, an irregular faint metachromasia which increased toward the superficial layers was observed. The presence of small metachromatic granules superimposed over the cells and the intercellular substance (ICS) of the granular layer was also observed. The keratin layer gave a constant metachromatic reaction. The presence of a zone of lighter metachromasia in the beginning of the keratin layer was noticed. Morphologically, this zone corresponds to the Stratum lucidum. The intercellular substance was observed to stain with a light metachromasia. This was always found in the upper spinous and granular layers; while in the germinal zone, irregular areas where the ICS was widened and without staining were also observed.

The basement membrane along the epithelium and connective tissue junction did not stain in both the normal and TX animals. (Fig. 9)

In the normal animals, the ground substance along the lamina propria presented a very light metachromasia. (Fig 1) Those areas where this staining reaction was observed usually surrounded the cells. In addition, interfibrillar spaces, scattered around the lamina propria gave a light metachromasia. The connective tissue fibers were outlined by unstained.

The nuclei and cytoplasm of fibroblast gave a metachromatic reaction with the nuclei staining more intensely. The presence of small metachromatic granules was observed in the cytoplasm of some of the fibroblasts. Mast cells were observed scattered throughout the connective tissue, but especially in the deeper submucosa layers. These cells were filled with granules that gave a very strong metachromatic reaction. (Fig. 7) The heavy concentration of these granules made it difficult to observe the individual cell detail. Also, it was observed that the great density of granules in some of the cells produced an apparent orthochromatic reaction. Closer examination revealed that the apparent orthochromasia was due to impeded light transmission, through the dense mass of granules. A number of these cells were found liberating their granules into the ground substance. These granules stained intensely and brilliantly metachromatic when seen scattered singly within the ground substance.

Endothelial cells also gave a metachromatic reaction; the nuclei were more intense than the cytoplasm and were similar, in this respect, to the

fibroblast.

In the lamina propria of TX animals, a more intense metachromasia was observed as compared to the normal animals. (Fig. 2) Large and more intensely stained metachromatic areas were distributed in a random manner about the ground substance. Such areas had a mucinous appearance and contained poorly outlined unstained fibers. Moreover, in such areas, a greater cellularity was noted as compared to adjacent, less intensely stained metachromatic areas. The predominant cell was the fibroblast, although Mast cells were also abundant. The presence of small metachromatic granules liberated from the Mast cells into the ground substance was also observed and were comparable to those seen in the normal animal sections.

The stainability of fibroblast, Mast cells, and endothelial cells was similar in normal and TX animals. The only difference that was observed was the randomly distributed loci of increased metachromasia in the ground substance of the lamina propria.

The skin of normal animals demonstrate metachromasia in the ground substance of the subcutis. The ground substance in the subcutis of thyroidectomized animals showed distinctively heavier metachromasia than observed in the skin of normal animals.

Thionin: *The metachromasia of the ground substance in TX animals was more intense than in the normal animals. However, it was evident that the overall degree of metachromasia obtained with the method was proportionally greater for both normal and TX animals than that obtained when Toluidine Blue was used. Apart from this increase in metachromasia in the ground substance the cellular components of the lamina propria stained in the same manner as with the toluidine blue method.*

Testicular Hyaluronidase: *In normal and TX animals, a reduction in metachromasia of the ground substance was observed following incubation for three hours with this enzyme. (Figs. 4 and 5)*

It was noticed that the model control slides of TX and normal animals incubated in buffer solution alone showed a reduction in metachromasia. This reduction was not as great as the one observed in the sections incubated in buffer plus enzyme. After treatment of the tissues with this enzyme, only a slight metachromasia remained in the ground substance.

The metachromatic reaction of the Mast cells granules, fibroblast and endothelial cells in TX and normal animals was not altered by enzyme treatment. Moreover, the granules liberated from the Mast cells into the ground substance maintained their same intense metachromasia.

The metachromasia found in the different epithelial components was not removed by testicular hyaluronidase.

Periodic acid Schiff: *There was no significant difference in the characteristics of PAS stainability between the normal and TX animals.*

In both groups, the only PAS positive area found in the epithelium was the intercellular substance recognisable within all the epithelial layers as a thin red line that disappeared in the keratin layer. The nuclei and cytoplasm of all the epithelial cells were PAS negative. When light green was used as counter stain, the epithelial cell nuclei and cytoplasm would accept the counter stain. The keratin layer was also PAS negative and consistently could be stained green.

By virtue of the wide distribution of available reactive polysaccharides, the connective tissue in both normal and TX animals gave a similar overall PAS positive reaction. In some areas, probably because of a greater thickness of the section, the PAS reaction was more intense.

The basement membrane between the epithelium and connective tissue and around capillaries stained very strongly PAS positive. (Fig. 8)

Mast cells cytoplasm were filled with small PAS positive granules. (Fig. 6) Some of these granules could be observed scattered in the ground substance. Occasionally, the thin cytoplasm of some of the fibroblast could be observed; and they likewise showed a PAS positive reaction. The

presence of small red staining granules in their cytoplasm sometimes could also be observed. Endothelial cells stained PAS negative.

Amylase: *Incubation with this enzyme did not modify the characteristic of PAS stainability of the epithelium in either the normal or TX animals. The intercellular substance continued to be the only PAS positive area present in the epithelium.*

The basement membrane did not visibly undergo modification following amylase incubation. Its intense PAS positive reaction remained the same.

The ground substance of the amylase treated specimens stained similarly to the model control slides, in both TX and normal animals. However, it was observed that after immersion in the buffer, with or without enzyme, the sections stained slightly less intensely than those stained without immersion in the buffer solution.

It was observed that after incubation with amylase, there was a loss of PAS positive granules from the Mast cell and fibroblast's cytoplasm. This was specially noticed in the Mast cells from the tissue specimens of TX animals. As a result from this enzymatic action, the Mast cells took a faded appearance making it more difficult to examine.

Gomori silver impregnation: *Along the basement membrane joining the epithelium and connective tissue and around blood vessels, the presence of*

argyrophilic fibers was constantly observed in both normal and TX animals. (Fig. 10)

In the lamina propria and deeper layers of the connective tissue, numerous rose-colored, mature collagen fibers were seen arranged in an irregular and intricate pattern. This finding was also similar in both normal and TX animals.

Heidenhain's azan stain (modification of Mallory's connective tissue stain):

An intense and regularly distributed blue stain was observed all over the intercellular spaces of the connective tissue, contrasting markedly with the red-stained cells and muscle fibers. Both reticulin fibers in the basement membrane and collagen fibers in the lamina propria stain with the same intensity, therefore, could not be individually identified with this method. The sections from normal and TX animals presented similar staining characteristics.

Haematoxylin and eosin: *The epithelium of the oral mucosa consisted of the usual basal, spinous, granular and keratin layers. The thickness of these layers varied in the different areas of the mouth, depending upon where these specimens were taken (e.g., The keratin layer in the masticatory mucosa is wider than in the lining mucosa). Regular intercellular spaces were observed along all the epithelial layers. The mitotic figures observed in the germinal layers of the epithelium of normal and TX animals*

were not found to be measurably different. The epithelium of TX and normal animals presented in all aspects, similar morphological characteristics.

The presence of fibroblast, Mast cells and macrophages were observed in the connective tissue. The overall number of these cells was similar in TX and normal animals. The absence of inflammatory cells in both normal and TX animals was striking.

The ground substance had an homogenous appearance and contained capillaries similar in number and structure in both normal and TX animals.

DISCUSSION

The histochemical methods generally used for the detection of acid mucopolysaccharides are based on the observation of the metachromatic shift that basic dyes experience when in contact with compounds with a net electrical negative surface (63). It has been clearly demonstrated that chondroitin sulfuric acid, hyaluronic acid and heparin stain metachromatically. A differentiation between these acid mucopolysaccharides can be done by their incubation with enzymes which will react specifically with some of them only. Thus testicular hyaluronidase will only hydrolyze hyaluronic acid and chondroitin sulfuric acid A and C; while heparin and chondroitin sulfuric acid B will not be affected (63). Subsequent staining of tissues containing such compounds can give us a picture of the localization and estimated amount of these substances in situ.

Braden (15) and Hoogwinkhel and Smits (35) have clearly demonstrated that acid mucopolysaccharides do not stain positive with the PAS method. Only neutral polysaccharides stain positively with this method. This offers us the opportunity to differentiate between neutral and acid polysaccharide tissue components, the acid mucopolysaccharides staining only metachromatically and the neutral polysaccharides PAS positive. If the tissues are

incubated with a specific enzyme, such as amylase, which removes the PAS positive glycogen, a further differentiation of the neutral polysaccharide fractions can be made. After incubation, only the remaining PAS positive fractions, such as glycoproteins, mucoproteins, and glycolipids will be stained. Therefore, the presence can be determined and an estimate made of the amount of glycogen in the tissue.

Watson and Pearse (77) demonstrated the existence of chondroitin sulfate and hyaluronic acid in normal skin; while Schultz-Haude (71) in a comprehensive study asserted the existence of these acid mucopolysaccharides in normal oral mucosa.

It is known that thyrotropic and thyroid hormones may have a direct action upon the connective tissue. In the case of hypothyroidism, one of the symptoms is the accumulation of abnormal amounts of a mucinous substance in the connective tissue of the skin of hypothyroid subjects. This pathological entity was denominated "myxedema". Watson and Pearse (77) studied the skin of myxedematous patients by biochemical assays and demonstrated that the mucinous substance was due to an increase in hyaluronic acid and chondroitin sulfuric acid. Asboe-Hansen (2, 3) also demonstrated histochemically an increase of these substances in the skin of myxedematous human subjects. Asboe-Hansen claimed that the larger quantity of acid mucopolysaccharide was hyaluronic acid. In the case of generalized

myxedema, this accumulation was found along the lamina propria while in pretibial myxedema, it was found in the deeper layers of the connective tissue. Asboe-Hansen (4) also described an increase of the mast cell population in these myxedematous areas and proposed the theory that these cells under the influence of TSH were responsible for the greater production of hyaluronic acid.

Del Conte et al (22) examined with biochemical and histochemical methods the skin of TX rats and reported findings similar to those described by Asboe-Hansen in the skin of hypothyroid humans; that is, the accumulation of metachromatic acid mucopolysaccharides and Mast cells in the connective tissue. The findings of Del Conte are supported by our observations of increased metachromatic mucopolysaccharides in the subcutis of our thyroidectomized rats.

Asboe-Hansen also believes that the Mast cells are solely responsible for the production of all the ground substance in the connective tissue. This concept has been justifiably attacked by K. Meyer (54) and Rothman (70) among others. Sylven (75) pointed out the difficulty for the proposed chemical re-structuration phenomena, that would have to occur in order to change the heparin contained in the Mast cells to the hyaluronic acid and chondroitin sulfate present in the ground substance. Also the overall number of Mast cells in the connective tissue does not seem to correlate

with the large content of ground substance. There are areas in the body where, in spite of the presence of large amounts of ground substance, no Mast cell can be observed. Furthermore, the fibroblasts, the most abundant cells in loose connective tissue, react similarly to the Mast cell by histochemical staining asserting the intracellular presence of neutral and acid polysaccharides make the idea of Mast cells as sole producers of ground substance, quite unacceptable.

The study of the connective tissue of the oral mucosa of normal and TX rats was carried on with the help of histochemical and histological methods similar to those employed by Asboe-Hansen, Del Conte and others for the detection of acid mucopolysaccharides, neutral polysaccharides and fibers components.

The most important finding was the increased metachromasia of the ground substance in the lamina propria of TX animals. This was demonstrated with both toluidine blue and thionin staining methods. This result gives support to the previously reported increased metachromasia in myxedematous skin, thus suggesting chemical alterations in the oral mucosa due to a decrease in the circulating thyroid hormones. However, in spite of this modification in the ground substance of TX animals, there were no observable clinical changes, such as ulcerations, desquamations, puffiness, etc., in the oral mucosa of TX animals. Their oral mucosa

had a macroscopic appearance similar to that in normal animals. Andersen and Asboe-Hansen (1) reported similar findings in nearly one hundred cases of hypothyroidism in humans. Even though the clinical picture of myxedema was absent, the skin showed more metachromasia than normal. Seemingly, there exists a direct relation between the state of hypothyroidism and the local accumulation of acid mucopolysaccharides in skin and oral mucosa. The special conditions that cause the appearance of the clinically visible myxedema in some cases and not in others are not known.

The decrease in metachromasia of the ground substance of the oral mucosa after incubation in testicular hyaluronidase suggests the presence of acid mucopolysaccharides of the order of hyaluronic acid and/or chondroitin sulfuric acid A or C. Pearse (63) stated that the chondroitin sulfate B is not hydrolyzed by this enzyme. The fact that some light metachromasia always remained in the tissues agrees with the findings of Schultz-Hauidt (71) who stated that the testicular hyaluronidase resistant chondroitin sulfate B is present in the connective tissue of the oral mucosa of man. A similar finding was observed with the metachromatic granules of the Mast cells. None of the intra or extra-cellular granules were affected by this enzyme. They stained in the same way before and after incubation with testicular hyaluronidase. The granules are, therefore, composed of a substance different than hyaluronic acid and chondroitin sulfate A or C.

In fact, it has been shown that the substance responsible for the strong metachromasia of Mast cells is heparin. In this regard, it is difficult to accept the idea that in myxedema the increased acid mucopolysaccharide content of the ground substance is the by-product of the heparin derived from the Mast cells. There was no observable increase in the overall number of Mast cells in the oral mucosa of TX rats. Mast cells are very abundant in the rat and we found a great number in the oral mucosa of normal animals. There were areas, especially in the deeper layers of the connective tissue, that showed intense Mast cell population in both normal and TX animals. Asboe-Hansen and Del Conte did not employ any precise method of Mast cell count in skin; therefore a quantitative statement about the number of these cells is unreliable.

There were areas of the ground substance, especially along the lamina propria of the attached gingiva, that gave a metachromatic reaction and in which the only cell available was the fibroblast. The cytoplasm of fibroblasts stain metachromatically. This fact suggests the direct participation of fibroblasts in the elaboration of the acid mucopolysaccharides components of the ground substance.

There was a very irregular pattern of distribution of areas of strong metachromasia in the connective tissue of TX animals. The reason for this random concentration of acid mucopolysaccharides in the tissue is not

known.

The results obtained with the PAS method showed no significant difference in the ground substance characteristics between the normal and TX animals. This suggests the presence in the tissues of both animals of similar amounts of reactive carbohydrate groups and also of their presence in a similar state of aggregation.

Engel (24) and Gersh and Catchpole (28) stated that an elevation in the water content of the tissue will cause a reduction in the intensity of the PAS reaction. They believe that the greater dispersion of tissue polysaccharides in a given area, cause by edema, will reduce the number of reactive groups available for the PAS to stain. However, the tissue specimens from the TX animals stained with the same intensity as that obtained from normal animals. This suggests the absence of edema in the connective tissue of the oral mucosa of TX animals.

The incubation of the tissues from normal and TX animals in a buffer solution followed by the PAS stain did not bring any significant reduction in the intensity of the reaction. This indicates that the greater number of heteropolysaccharide complexes in the connective tissue of a normal rat are, because of their state of aggregation, of a water insoluble nature; and, in the same manner, that thyroidectomy did not cause modifications observable with the methods used by us.

The Mast cells were observed to stain positive with the PAS and metachromatic methods; therefore, we can assert the presence in these cells of neutral and acid polysaccharides. Similar results were obtained in the fibroblasts. This supports the concept that both fibroblast and Mast cells play an important role in the production of ground substance.

The most interesting result observed after the incubation of normal and TX tissue specimens in amylase was the marked hydrolysis of many of the PAS positive granules present in the cytoplasm of Mast cells. This was more evident in the Mast cells of TX specimens. The remaining PAS positive substances in the cytoplasm of Mast cells are heteropolysaccharides of a different nature than glycogen. The larger content of glycogen in the Mast cells of TX animals might be related to their greater production of ground substance. Mosbach and King (60) and Roseman et al (69) have demonstrated that glucose can be used as a primary structural unit in the synthesis of acid mucopolysaccharides. The role of Mast cells in the synthesis of acid mucopolysaccharides has been discussed by Asboe-Hansen. However, he believes that the production of hyaluronic acid is by way of a heparin-like precursor. Sylven (75) stated that from a chemical and structural point of view, it is very unlikely that this change would occur. But, if instead of a heparin precursor, glycogen is utilized, as suggested by Mosbach and King (60) and Roseman et al (69), the role of Mast cells in

the synthesis of acid mucopolysaccharides could be more easily understood. The higher content of glycogen observed in the Mast cells of TX animals, suggest the possibility of their increased synthesis of acid mucopolysaccharides. However, it could be attributed also to the intra-cellular retention of larger amounts of glycogen, because of low metabolic requirements.

The basement membrane along the epithelium and connective tissue junction appeared similar in both normal and TX tissue specimens. It was observed that the main constituents of this particular zone were the reticulin fibers and the PAS positive glycoproteins. It was interesting to notice that the basement membrane did not show any metachromasia indicating the absence of acid mucopolysaccharides in this particular zone.

No significant difference was observed with the Gomori silver impregnation and the Heidenhain's azan stain methods in the collagen components of the connective tissue of normal and thyroidectomized animals. The fibers were observed to be normal in number and structure in the oral mucosa of TX rats. This finding is contrary to the Ziskin and Stein (82) report of collagen degeneration in the oral mucosa of TX Rhesus monkeys.

An interesting observation was the absence of inflammatory cells in the connective tissue of TX animals. The cellular components of both normal and TX animals were observed to be similar. This finding is contrary to the report of Baume et al (11) who found a reduction in the cell

population of the connective tissue of the oral mucosa of TX rats.

The epithelium of the oral mucosa of normal and thyroidectomized animals did not show any significant difference with any of the methods used. We were not able to observe any degenerative changes in the epithelium of TX animals, as was reported by Baume and Ziskin and Stein.

The only definite alteration that was brought about by thyroidectomy was the increase accumulation of acid mucopolysaccharides, especially the hyaluronic acid type in the ground substance of the connective tissue of TX animals. No other signs of pathology were detectable in the tissues under study, with the methods used by us.

SUMMARY AND CONCLUSIONS

An experiment to study the possible alterations of the connective tissue in the oral mucosa of thyroidectomized rats was done. Special attention was given to the study of the mucopolysaccharide components of the ground substance of the connective tissue. A comparative evaluation of the oral mucosa from TX and normal animals was done.

Thirteen male albino rats were used. Nine of these animals were Thyro-parathyroidectomized at the age of four weeks, while the remaining four normal animals were used as control. The animals were sacrificed at the age of seven months.

Tissue specimens were obtained from the oral lining and masticatory mucosa and skin. The tissues were treated by either the freeze-drying method or alcohol-acetic acid and acridine-ethanol fixative solutions.

Different histochemical stainings for the determination of mucopolysaccharides were used: Toluidine Blue, Thionin and PAS. Also, the following histological methods were used to study the fiber components and general morphology of the connective tissue: Gomori silver impregnation, Heidenhain's azan stain, and haematoxylin and eosin. Enzyme incubation in Testicular Hyaluronidase and Amylase was also done.

The results lead us to the following conclusions:

- 1. Thyroidectomy causes alterations in the connective tissue of the oral mucosa of the rat similar to those that have been previously described in skin. This alteration was in the form of a moderate increase of acid mucopolysaccharides, especially hyaluronic acid, in the ground substance of the connective tissue of TX rats.*
- 2. The oral tissues did not present any clinically visible sign of "myxedema".*
- 3. There was a greater content of amylase removable glycogen in the Mast cells of TX Animals than in normal animals.*

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**Fig. 1. Normal rat. Oral Mucosa. Very Light Metachromasia in
Connective Tissue. Freeze-drying. Toluidine blue stain, pH 7 x 100**

**Fig. 2. Thyroidectomized rat. Oral Mucosa. Metachromasia in
Connective Tissue. Freeze-drying. Toluidine blue stain, pH 7 x 100**

Figure 1.

Figure 2.

Fig. 3. *Thyroidectomized rat. Oral Mucosa. Metachromasia in Lamina propria of Connective Tissue. Mucoid appearance of the Ground Substance Freeze-drying. Toluidine blue stain, examined under water, pH 7 x 450*

Figure 3.

Fig. 4. Thyroidectomized rat. Oral Mucosa. Metachromasia in Lamina Propria of Connective Tissue. Freeze-drying. Sorensen's buffer incubation, three hours. Thionin stain, pH 7 x 450

Fig. 5. Thyroidectomized rat. Oral Mucosa. Greatly reduced metachromasia of ground substance after incubation in testicular hyaluronidase for three hours, 37 C. Freeze-drying. Thionin stain, pH 7 x 450

Figure 4.

Figure 5.

Fig. 6. *Thyroidectomized rat. Oral Mucosa. Mast cells liberating their granules into the ground substance. Freeze-drying. Toluidine blue stain, pH 7 x 1000*

Fig. 7. *Thyroidectomized rat. Oral mucosa. Mast cell containing PAS positive material in its cytoplasm. Freeze-drying. Periodic-acid Schiff stain. x 1000*

Figure 6.

Figure 7.

Fig. 8. *Thyroidectomized rat. Oral Mucosa. Basement membrane along the connective tissue and epithelium junction. Very strong PAS positive reaction. Freeze-drying. Periodic-acid Schiff stain. x 450*
(Notice the PAS positive reaction in the ICS of the epithelium)

Fig. 9. *Thyroidectomized rat. Oral Mucosa. Basement membrane along the epithelium and connective tissue does not show metachromasia. Freeze-drying. Toluidine blue stain, pH 7 x 1000*
(Notice the metachromasia in nuclei and ICS of the epithelium)

Figure 8.

Figure 9.

Fig. 10. Thyroidectomized rat. Oral Mucosa. Basement membrane along the connective tissue and epithelium junction. Gomori silver impregnation x 450

Figure 10.

APPROVAL SHEET

The thesis submitted by Dr. Rodrigo Eisenmann Grimas has been read and approved by three members of the Department of Anatomy and Oral Biology.

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.

May 23, 1962
DATE

Signature of Adviser