1978

Insulin I-p1-s-p2-s-p5-s Distribution within Oral Tissues

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INSULIN I^{125} DISTRIBUTION

WITHIN ORAL TISSUES

by

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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

1978

DEDICATION

I dedicate this thesis to my father and my mother, Mr. and Mrs. Pourdeihimi, whose encouragement, understanding and their lasting patience and great sacrifices made in order to further my education.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Joseph J. Keene Jr. for his personal interest, guidance and direction during the preparation of this thesis.

I wish to thank Dr. Anthony W. Gargiulo and Dr. Patrick D. Toto for their unlimited patience, guidance and providing me the opportunity to study in periodontics at Loyola University.

Finally, I would like to thank Dr. James P. Filkins, chairman of Physiology, Loyola Medical School, for his helpful suggestions and continuous assistance and also wish to express my thanks to the laboratory staff within the Department of Oral Pathology, School of Dentistry, for most helpful assistance.

LIFE

The author, Bahram Pourdeihimi, is the son of Habib Pourdeihimi and Tooran (Tabatabaei) Pourdeihimi. He was born on May 10, 1945, in Kermanshah, Iran.

His elementary education was obtained in Hedayat School and secondary education at Pahlavi High School in Kermanshah from which he was graduated in June, 1964.

In September, 1965, he entered National University of Iran School of Dentistry in Tehran where he received the degree of Doctor of Dental Medicine in June, 1971.

He then entered the Iranian Army and served as a dentist in the Regional Army Hospital in Kermanshah for a period of two years.

In the fall of 1975, he entered Loyola University School of Dentistry in a three year graduate program leading to a certificate of specialty in periodontics and Master of Science in Oral Biology.

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CHAPTER I

INTRODUCTION

Diamare (1889) and Laguesse (1893) were probably the first to suggest that the islet tissue is concerned in the production of an internal secretion whose function is the control of carbohydrate metabolism.[18]

In 1921 Frederick G. Banting and Charles H. Best,[31] working in the Laboratory of J.J.R., Macleod in Toronto, first extracted the insulin hormone and experimentally showed insulin lowered hyperglycemia and glycosuria within the diabetic animal. Later, in 1922 Macleod's studies[18] definitely established the fact that insulin is elaborated by the islet tissue. The purpose of this investigation is to evaluate and compare the distribution of insulin within the following oral tissues: skeletal muscle,, glandular tissue, connective tissue, bone, epithelium, dentin and the dental pulp. Control tissues will consist of liver and thryoid.

The study is undertaken in order to achieve a greater understanding of the physiology of insulin within oral tissues. To accomplish this goal the experimental animals are chemically made diabetic with streptozotocin and insulin I^{125} is utilized as part of insulin replacement therapy for the identification of insulin distribution.

Experimental animals will be made diabetic with streptozotocin

in order to obtain an increased uptake of insulin I^{125} within evaluated oral tissues. It is hoped that the tracer can be utilized more effectively since endogenous insulin uptake will be minimized due to the induction of the diabetic state.

CHAPTER II

REVIEW OF THE LITERATURE

EMBRYOLOGY OF PANCREAS ISLETS

Embryologically both the exocrine and endocrine portions of the pancreas develops as buds from cords of cells growing out of the ducts from the intestine and the hepatic diverticulum.

The islets lose most of their connections with the duct system after the third month in utero, and insulin production begins at this time. The blood supply of the pancreas arises from the splenic, hepatic, and superior mesenteric arteries and veins.

ANATOMY AND HISTOLOGY OF PANCREAS ISLETS

The islets of Langerhans, constitutes only about 1 to 2 percent of the total weight of the Pancreas,[23] but there are up to two million islets, varing in diameter from 20 to 30 u.

In 1926 Ukai, differentiated two distinct types of cells in the rabbit islet, Alpha and Beta cells.[31] Lacy and Williamson in 1960[21] demonstrated endogenous insulin in Beta cells by the fluorescent antibody technique. The Alpha cells secrete glucagon which was discovered in 1920 by Murlin.[33] In 1931, Bloom[31] observed, a third type of cell in the human pancreas which he termed Delta cell; a specific function for this cell has not been described.

Bensley[18] (1889) has described a nongranular cell type in the

guinea pig, it has been termed C-cell, and may possibly be a precursor of the Alpha cell.

LIGHT MICROSCOPIC STUDIES OF ISLETS

Under light microscopic examination, the typical Beta cell is many sided and irregular in outline. The Alpha cell is also irregular but is somewhat elongated and larger in size.

A method for Alpha, Beta and Delta cell differentiation was devised by Gomori[16] (1950), utilizing chrome alum hematoxylin phloxine.

In this procedure, the granules of the Beta cell stain a deep blue from the chrome hematoxylin, and rather coarse, and their number per cell varies greatly. The granules are not distributed evenly throughout the cell and are often densely packed in a portion of the cell. In contrast, the Alpha cell granules stain bright red with phloxine and are finer than the granules of Beta cells. They are also more numerous and more evenly distributed within the cell. The Delta cell contains small blue stained granules. The islet consists of 60-70% Beta cells, 20-30% Alpha cells and 2-8% Delta cells.

ELECTRON MICROSCOPIC STUDIES OF ISLETS

Lacy[22] (1961) and others have described electron microscopic observations of the islets. Individual Alpha and Beta cells are enclosed by a distinct, continuous plasma membrane and their nuclei are surrounded by a double membrane. The Cytoplasm of these cells contains rough endoplasmic reticulum, mitochondria, granules and portions

of golgi complex. The rough endoplasmic reticulum consists of the lamellar membranes and their associated small cytoplasmic granules. The rough endoplasmic reticulum within the Beta cells is apparently the base for the secretion of the granules,[22] which contain multiple proteins including insulin. Therefore, the endoplasmic reticulum is considered to be the site of protein synthesis. Insulin is one of the proteins synthesized within the endoplasmic reticulum of the Beta cell.

PHYSIOLOGY OF INSULIN

Insulin is secreted by a process known as emiocytosis.[31] Defined, emiocytosis is the secretion of insulin into the blood stream by passing through the cellular spaces and membranes before entering the blood stream.

Stimulation of Beta cells cause migration of insulin to the surface of the Beta cells. The mechanism for this action is hypothesized that the granules of the Beta cell migrate through the cytoplasm to the plasma membrane where they fuse together upon contact and rupture to the exterior surface of the cell. The insulin molecules then pass through the intercellular fluid to the lumen of the capillary where it is transported via the blood stream to certain tissues.

Zinc is found in high concentrations within the Beta granules and is believed to be essential for holding insulin in the granules of Beta cells. Likewise, zinc bound in insulin increases the solu-

bility of insulin outside the cell.[32] The major factors stimulating insulin secretion in vivo are hyperglycemia, fatty acids and amino acids. The latter lead to Beta cell degranulation.

INSULIN RECEPTOR SITES

"Receptor" signifies those molecules of the cell membrane which are uniquely capable of recognizing and interacting with insulin and which, in addition, possess the capability of metabolic events. The receptor has at least two functions:

1. To specifically recognize insulin.
2. To convey to other molecules that insulin has been recognized.

Studies indicate that the "insulin-cell receptor interaction" is in the external surface of the cell membrane,[17] so the initial and singular locus of insulin action is the cell membrane, Fig. 1. Direct evidence for the exclusive localization of insulin receptors to the surface of the cells has been obtained.[8] Recent studies indicate that the hormone need not enter the target cells to exert its action and that a very superficial and brief contact with the plasma membrane may be sufficient to elicit its effect.[17] A maximum of about 11,000 molecules of insulin can bind per cell.[8] Fat cell membranes are believed to have the highest insulin binding capacity. A number of studies have indicated that the properties of the insulin receptor interaction in the isolated membrane preparations are almost identical to those observed with intact cells.[17]

In vivo, purified liver membrane has many properties which are almost identical to those of fat cell membranes, suggesting that the receptor structures may be similar or identical in these two tissues.[8] The possible contribution of membrane carbohydrate, protein and lipid structures to the integrity and function of the insulin receptor has been studied.[8]

ACTION OF INSULIN

Insulin may act in the following fashion:

1. The insulin molecule attaches to a specific receptor at the cell membrane.
2. This event causes specific changes in the molecular assembly pattern of parts of the cell membrane.
3. These changes in turn activate the transport carriers for certain sugars, amino acids, and electrolytes, and at the same time a small molecule is released into the cell as a "second messenger".
4. The second messenger must then act upon a reaction shared at some point by the processes of anabolism.[25]

The inhibitory activity of insulin is apparently independent of the manner by which the enzyme is stimulated. Inhibition by insulin can be observed in the enzyme stimulated by epinephrine, glucagon, A.C.T.H. and sodium floride.[17]

INSULIN SENSITIVITY

During fetal life the basal rate of glucose uptake gradually

decreases and sensitivity towards insulin develops rather late, apparently coinciding with the appearance of insulin in the pancreas. The presence of insulin receptor sites is not necessarily associated with insulin sensitivity towards glucose transport.[17]

Newerly and Berson[27] (1957) have shown that insulin binds to non-living material such as, glass, nylon, rods, talc, and also the hormone has a great propensity to adhere to proteins such as albumin and may also occur with a variety of cellular structures.[15 - 17] Worthington, Jones, and Bues[34] (1964) have shown that insulin binds to elastic tissue which is not known to be insulin sensitive. Therefore, insulin binding appears to be a rather general phenomenon and may not be solely a physiological one. The interaction between lipid and carbohydrate metabolism may be of importance for carbohydrate tolerance and insulin sensitivity. Longer exposure to fatty acids or their degradation products may cause a more lasting reduction in insulin sensitivity.[17]

Among mamalian tissues, insulin produces anabolic effects[19] in adipose tissue, cartilage, liver, mamary gland (some species), muscle, skin, uterus, seminal vesicle, diaphragm[15] and ciliarybody;[24] but not in aorta, brain, intestine, or lymphoid tissues.[19]

The role of insulin as a stimulator of the growth of fibroblasts is well documented.[17] It is clear that insulin is necessary for the growth of different tissues similar to the necessity of serum for tissue growth.[17] For example in insulin deficiency fibroblasts

are not stimulated for the healing process, so growth and the repair of damaged tissues are retarded. Many investigators believe insulin reduction is associated with loss of biologic activity within specific tissues.

THE CHEMISTRY OF INSULIN

Insulin hormone is a protein consisting of fifty-one amino acids and having a minimum molecular weight of 6,000. It is quite stable in dilute acid solutions at a P.H. range of 2.5 to 3.5. Insulin is relatively insoluble within the P.H. range of 4 to 7. For crystallization of the hormone it is necessary to add zinc or other metal ions such as nickel, cobalt, or cadmium.

The insulin molecule is composed of two polypeptide chains, designated "A" and "B". Both chains are connected by two interchain disulfide bridges of cystine; chain "A" also contains a disulfide intrachain. Pig, dog, and human insulin have a similar amino acid 8, 9, 10, "A" chain and differ only in amino acid 30 of the "B" chain. Pig and dog have Alanine and human have Threonine in amino acid 30 of the "B" chain. Beef insulin as compared with human insulin is different in amino acid 8 and 10 of the "A" chain and amino acid 30 of the "B" chain. Beef insulin contains amino acid 8 (Alanine) and 10 (Valine) within the "A" chain, as compared with human insulin in which amino acid 8 is Threonine and 10 is Isoleucine, Fig. 2. Amino acid 30 (Alanine) within the "B" chain of Beef insulin is replaced by Threonine similar as described for pig insulin.[31]

PRO-INSULIN

Steiner and Oyer[30] (1967), at the University of Chicago, treated insulin with trypsin and found a larger molecule than insulin. They concluded that the larger molecular weight material was proinsulin.

At about the same time, Chance, Ellis, and Bromer[5] (1968), working with procine insulin, also found a large molecular-weight material which appeared as a single band. This fraction (proinsulin) consisted of about 1 percent of the insulin material and had a biological and immunologic activity approximately 10 to 20 percent that of insulin. Further research showed that its molecular structure consisted of the "A" and "B" chains of insulin plus thirty-three amino acids. Proinsulin degraded less rapidly than insulin and because of the large molecular size inhibited the binding of insulin within tissue.[14]

INSULIN ANTIGENICITY

The immunological determinants of insulin are located at the carboxyl end of the "B" chain and in positions 8,10, of the "A" chain. Dog, pig and whale insulin have identical amino-acid sequences. Human, pig, dog, cow, sheep, rabbit, and rat insulin and that of other animals differ in their amino acid structures and therefore react differently with an anti-insulin serum. Modifications of the insulin molecule by amino acid substitution, by elongation or shortening of the chains at the chain termini show that changes in the insulin molecule decrease antigenicity.[17]

Berson and Yalow[3] (1962), have hypothesized that insulin com-

bines only with one molecule of antibody.

FREE AND BOUND INSULIN

Insulin in the blood circulates in two forms called free and bound. Free insulin is similar to crystalline insulin. It is released by the pancreas under glucose stimulation, it is biologically active, and reactive with anti-insulin antisera, its molecular weight is about 12,000.

Bound insulin which is bound to glyco or mucoprotein may represent an inactive metabolite of free insulin. The transformation of free to bound insulin being catalyzed in vivo by the liver and possibly by other extra pancreatic tissues, its molecular weight is between 40,000 and 60,000. Bound insulin is unreactive with anti-insulin antisera.[1]

INSULIN DEGRADATION

It has been demonstrated by Mirsky, Elgee and others that insulin is rapidly inactivated both in vitro and in vivo. Essentially all tissues of the body can inactive insulin. Degradation can be both enzmatic and non-enzmatic.[32] Insulin degradation was detected in all tissues by Chandler and Varandani,[6] (1972). The relative activities were to be in order; pancreas > liver > intestine>spleen > kidney, testis, thymus, fat, lung > brain > heart > diaphragm, skeletal muscle. Insulin degradation was completely blocked by the addition of N-ethyl-maleimide. These investigators suggest that gluta-

thione-insulin-transhydrogenase may be the major insulin-degrading activity present in the animal.[6] Insulin degrading system is located on the external surface of the plasma-membrane of the cells.[17]

INSULIN I^{125} DISTRIBUTION WITHIN TISSUES

The biologic activity of insulin has been demonstrated by chromatography, autoradiography, sentillation counting, or combination of such procedures applied to radioactive isotope techniques.

This portion of the literature review is directed to the use of insulin I^{125} as a responsible method of analysis for insulin distribution or action. Garatt, Jarrett, and Keen[15] (1966), investigated I^{131} (from I^{131} iodoinsulin) and I^{125} (from I^{125} iodoalbumin) association and action within rat retina, diaphragm and epidermal fat. They found that the rates of association of I^{131} and I^{125} with all three tissues were initially rapid and similar, but after 5-10 minutes of exposure, the level of I^{125} reached a relatively constant rate. Likewise Beck and Fedynskyj,[2] (1976), utilized insulin I^{125} in order to study insulin concentration within the mouse liver (Kupffer cells), kidney cortex and the abilities of the glomeruli to filter labelled insulin molecules as well as the proximal tubule cells to reabsorb these labelled insulin molecules.

In 1969, Larsen and Werner[24] utilizing insulin I^{125}, showed grain accumulations due to insulin-insulin antibody interaction in the human non-diabetic and diabetic specimens of the eye. The insulin I^{125} reaction was most pronounced in the endothelial lining basement

membranes and walls of the vessels in the ciliary body, iris, retina, and choroid, as well as in the retrobulbar fat.

In 1973, Gammeltoft and Gliemann,[14] studied the binding and degradation of insulin I^{125} by isolated rat fat cells. Their findings were as follows:

1. The concentration dependence of equilibrium binding of I^{125} labelled insulin showed that one part of the binding could be saturated, where as another part was proportional to the concentration in the range investigated. Neither vasopressin, glucagon, nor des- B23-B30-octapeptide insulin, reduced the binding of I^{125} labelled insulin, where as proinsulin inhibited the binding.
2. Insulin I^{125} in the medium was degraded by fat cells. Proinsulin was degraded less rapidly than insulin and the degradation of insulin I^{125} was inhibited by proinsulin, where as glucagon had no effect.
3. The results show that the principle binding of insulin by fat cells occurs to a group of receptors, and that the degradation of insulin and the receptor binding are processes independent of each other.

CHAPTER III

MATERIALS AND METHODS

Eleven Sprague-Dawley (Holtzman) male rats weighing from 211 to 335 grams were utilized in this study.

Eight of the rats were injected Intraperitoneal (I.P.) with 65mg/kg Streptozotocin* diluted in a 1 c.c. citrate buffer solution. Each animal was injected within five minutes of the solution preparation. The single injection of Streptozotocin in solution normally made the animals diabetic within 1 week.

Citrate buffer solution:

Preparation of the citrate buffer solution was as follows:

Sol. A-1.114 gram citric acid H_2O was diluted with 100ml. NaCl 0.9%.

Sol. B-1.382 gram Na citrate 2 H_2O was diluted with 100ml. NaCl 0.9%.

Solution A was mixed with Solution B until the PH of the Citrate Buffer Solution was 4.5.

Care was taken to insure good asepsis at the time of injection.

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*Streptozotocin: Lot No. 60140, U-9889, was obtained from Dr. W. Dulin, The Up John Company, Kalamazoo, Mighigan.

Gloves were worn also to protect the investigators against the Streptozotocin which has shown to produce pancreatic tumors in experimental animals.

The diagnosis of diabetes mellitus was made when the animal gave evidence of the following, Figs., 3,4:

A) Weight loss or absence of normal weight gain during growth period.

B) Polydypsia and polyuria (Table 1).

C) Glycosuria (Table 2).

D) Hyperglycemia (Table 2).

After one week each experimental animal was injected with 20 u of commercially prepared insulin I^{125}*, which was diluted in 1cc of distilled water.

INSULIN I^{125}

The insulin I^{125} was shipped lyophillized in sodium phosphate buffer (P.H. 7.5), containing a stabilizer and a proteolytic enzyme inhibitor. The isotope was reconstituted as directed within the technical specifications of the laboratory. Specific activity, chemical purity, immunoreactivity and extent of iodination was determined by New England Nuclear.

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*Nex - 104 Insulin (I^{125}) Porcine, monoiodinated
New England Nuclear, 549 Albany St., Boston, Mass.

INJECTION OF ANIMALS WITH TRACER:

The animals were injected I.V. with insulin I^{125} through the dorsal vein of the penis.

Eight rats with Streptozotocin induced diabetes and two non-diabetic (i.e., not injected with the diabetic inducer) were injected I.V. with insulin I^{125}.

One rat acted as a complete control and was not subjected to either the diabetic inducer or I.V. insulin I^{125} administration. Fig. 5.

Experimental rats were sacrificed by ether at the time intervals of 2,5,9,12,15,28 minutes and 4,5,7 hours. Immediately upon sacrifice the following structures were dissected: thyroid, liver (six animals), tongue, cheek, hard palate, floor of the mouth, and mandibular central incisors with attached gingivae. All of the tissues were immediately fixed in 10% formalin and prepared for histologic sectioning.

The anterior portions of the mandibles were placed in formic acid for a period of 12 days for decalcification prior to fixation. Decalcification was confirmed by the x-ray.

Specimens were brought through alcohol, xylenes, embedded in paraffin and sectioned with the microtome approximately 5 u in thickness, floated on glass slides, dryed and deparaffinized according to conventional histologic technique in preparation for the autoradiographic procedures.

AUTORADIOGRAPHIC PROCEDURE:

The tissue sections were carried through Kodak NTB_2 emulsion in the dark room. The emulsion which was stored in the refrigerator at 4°C was placed in a warm bath at 43°C for approximately one hour and then poured into a glass container previously warmed to 43°C. Each slide was dipped into the melted emulsion for one second. The slides were dried by standing them on a bias on a wet tissue paper placed on the base of a wood drying rack. They were allowed to dry for 1 hour. The dried slides were then placed in black boxes with lithium chloride (i.e., absorb water) sealed and kept in the refrigerator for 22 days. On the 22nd day, the exposed slides were developed in Kodak developer (18°C) for 3 minutes, washed in distilled water (18°C) 3 times and then placed in Kodak fixer at 18°C for 3 minutes. After fixation the slides were placed under 18°C running water for 15 minutes.

The developed and washed slides were then stained with hematoxylin for background structure. The stained slide specimens were carried through acid alcohol, alcohol, xylene and covered with permount. Control hematoxylin and eosin slides were made of each tissue.

All diabetic and non-diabetic specimens injected with insulin I^{125} were viewed and evaluated under the light microscope for grain distribution. The micrometer 10mm., 100 interval eyepiece was utilized to make approximate grain counts.

The grain count was made utilizing the following squares:

A) upper left four squares

B) upper right four squares

C) lower right four squares

D) lower left four squares

E) middle four squares

The data was subjected to the two way block analysis of variance. (Table 7). Fig. 26,27.

The mean value (Table 6) for individual tissue counts and K distribution for analysis of tissue significance was calculated, Fig. 28.

CHAPTER IV

RESULTS

The distribution of radioactivity was demonstrated by grain counts within the multiple tissue preparations. Throughout this study both the control and diabetic specimens showed a greater grain count than the background.

THYROID

The uptake of I^{125} within thyroid follicules elevated rapidly from 5 to 9 minutes. The greatest grain count of I^{125} within the thyroid follicules was observed in 15 minutes. Between 15 minutes and seven hours the I^{125} value remained high. Table 3. The latter uptake of I^{125} by the thyroid is believed to indicate the degradation product of insulin I^{125}, permitting the I^{125} to be incorporated into thyroglobulin (thyroxin). Figs. 6,7,8 & 9.

LIVER

The maximum uptake of insulin I^{125} was from 12 to 15 minutes within the rat liver. Table 3. The insulin I^{125} appeared localized within the connective tissue and sinusoids in close relation to the Kupffer cells or bound to the plasma membranes of liver cells. Figs. 10 & 11.

SKELETAL MUSCLE

The labelled insulin I^{125} seemed associated with muscle Plasma

membrane and intercellular substance. Figs. 12 & 13. In cross section the grains were distributed evenly throughout the intercellular substance between muscle fiber bundles. Fig. 14. The grain count for muscle was low compared with other tissues evaluated. This count could be related to the dosage of insulin I^{125} utilized in this study, since Stein and Gross[29] (1959), found that with increasing insulin I^{125} dosage the ratio of insulin I^{125} concentration in the rat kidney and muscle tends to increase.

BONE

The uptake of insulin I^{125} in bone autoradiographically appeared to be relatively high. However, this apparent uptake by the tissue is questionable. The actual insulin count in bone may be low since the vascular spaces of the bone marrow contained abundant insulin I^{125}. Figs. 15 & 16.

DENTAL PULP

An unexpected finding was the high insulin I^{125} autoradiographic grain count within the pulp chamber of the rat incisor. This grain count is believed to be associated with the vasculature and connective tissue of the pulp chamber. Figs. 17 & 18.

PERIODONTAL LIGAMENT

Insulin I^{125} grains were consistently noted within the connective tissue of the periodontal ligament. This grain count within the periodontal ligament appeared high when compared to the other tissues. Figs. 19 & 20.

ORAL EPITHELIUM

The distribution of insulin I^{125} grains within the hard palate epithelium was primarily found within the basal cell layer and stratum spinosum. The insulin I^{125} grains were seen associated with the area of the epithelial basement membrane and intercellularly between adjacent epithelial cells. The stratum granulosum presented a grain count only slightly higher than that of the background. No distribution of grains were observed within the stratum corneum. Figs. 21 & 22.

CREVICULAR EPITHELIUM

Similar to oral epithelium, the crevicular epithelium showed insulin I^{125} grains distributed primarily within the basal cell and spinous cell layers which greatly diminished within the granular layer. The keratinized crevicular epithelium of the rat generally showed no deposition of grains within the corneum. Fig. 23.

OTHER TISSUES

Observations of connective tissue, epithelial attachment and glandular tissue presented a low to negative grain distribution when compared with the background counts. Figs. 24 & 25. In all cases the grain count for dentin was equal or less than the background count. Fig. 19.

CONTROLS

The two non-diabetic experimental control rats injected with insulin I^{125} sacrificed at 2 and 9 minutes showed the same grain accumu-

lation pattern as observed within the diabetic experimental animals. However, the grain distribution appeared lower than the diabetic rats.

STATISTICAL ANALYSIS

Because insulin I^{125} degradation in the rat occurs within minutes only the 5,9,12,15, and 28 minutes grain count values were utilized for statistical data. The results of the grain counts differentiating between the tissue sections and the animals were as follows:

1. Accumulation of radioactivity was statistically significantly higher in the thyroid tissue of all specimens than the other tissues studied. According to the two way block analysis of variance and K distribution there was no difference of grain counts between tissue specimens within experimental animals with the exception of the thyroid. Table 3. Fig. 26 & 28.
2. There was no significant difference between experimental animals. Table 7. Fig. 27.
3. In order to approximate insulin I^{125} distribution within each tissue type, the mean of all experimental grain counts were calculated and presented in table 6. From this data observations can be made as to the high and low grain counts among the various tissues evaluated.

CHAPTER V

DISCUSSION

The accumulation of I^{125} was present in oral tissues, liver, and thyroid. In tissues which gave a positive autoradiographic grain distribution with the exception of thyroid, the positive findings are believed to indicate the presence of insulin I^{125}. In this investigation experimental rats were made diabetic chemically with Streptozotocin and the insulin I^{125} was utilized as a part of insulin replacement therapy.

Dulin and Wyse[10-11] (1969), postulated that Streptozotocin may interfere with NAD (Nicotinamide Adenine Dinucleotide) formation in the Beta cells which results in decreased insulin synthesis and secretion rendering the animal diabetic.

Insulin I^{125} was utilized because it is a low energy γ emitter and has a T 1/2 of approximately sixty days, thereby permitting increased total working time. Worthington et al.,[34] in their study compared insulin I^{125} to insulin I^{131} and found insulin I^{125} to secure sharper autoradiograms. In their study autoradiography was utilized for identification of insulin I^{125} in tissues. Worthington et al.,[34] believe that autoradiography is the most promising technique for this task.

The increased grain counts of I^{125} within the thyroid versus time is believed to indicate the degradation product of insulin I^{125}

with resultant thyroid uptake of the I^{125}. Because of the thyroid uptake of I^{125}, the results of this paper would indicate optimum insulin activity occurs in the rat from 9 to 15 minutes. Accumulation of I^{125} in the thyroid remained high even after 7 hours past injection of insulin I^{125}.

The localization of radioactivity within the liver was in the connective tissue of the liver in close relation to the liver cells (Kupffer cells). This supports the previous reports by Beck and Fedynskyj[2] and Worthington et al.[34]

The low grain counts in skeletal muscle is believed related to the dosage of insulin I^{125} as discussed previously within the context of the result.[29]

The relatively high value of radioactivity which appeared within bone tissues most probably did not indicate insulin binding at that site but rather was associated with the bone marrow vasculature.

The distribution of radioactivity within the pulp was high. This finding seemed to be related primarily to the abundant blood circulation in the blood vessels of the pulp.

Heavy accumulations of radioactivity occurred in the periodontal ligament. These may have been due to the continuous eruption of the rat central incisor which histologically is associated with increased fibroblastic activity and collagen polymerization.

Epithelium evidenced the highest grain counts within the intercellular substance of the basal cell layer and stratum spinosum. It

is interesting to compare this distribution of insulin I^{125} with the findings of glycogen within the same cell layers,[12-13] since both of the latter appear to be involved in epithelial metabolism. Several sections evidenced a high basement membrane uptake of insulin I^{125}. Larsen and Werner[24] in their experiment found pronounced density of radioactivity in the basement membrane of the ciliary body, iris, retina and choriod.

Labelled insulin I^{125} was found in a high distribution within the gingival sulcus. This finding may be related to the passage of the isotope through the intercellular substance of the keratinized crevicular epithelium outward into the gingival sulcus. Ratcliff[28] (1968), reported the passage of microscopic size carbon particles from vasculature through connective tissue and intercellular epithelium into the keratinized gingival sulcus of the Holtzman rat. Otherwise the labelled intrasulcular insulin I^{125} may be related to salivary flow or artifact. Fig. 23.

CHAPTER VI

CONCLUSIONS

The localization of insulin I^{125} in oral tissues, thyroid and liver of the rat was investigated.

Eleven male Sprague Dawely rats were utilized in this study. Eight of the rats were made diabetic chemically with Streptozotocin. The diagnosis of diabetes mellitus was confirmed by polydypsia, polyuria, glycosuria, hyperglycemia and loss of weight. After one week, eight of the diabetic rats and two control non-diabetic rats were injected I.V. with 20 u of insulin I^{125}. One rat acted as a complete control and was not subjected to either the diabetic inducer or I.V. insulin I^{125} administration. Experimental rats were sacrificed at the time intervals of 2,5,9,12,15,28 minutes and 4,5,7 hours.

Immediately upon sacrifice the thyroid, liver, tongue, cheek, hard palate, floor of the mouth, mandibular central incisors with attached gingiva and the anterior portion of the mandible were dissected and fixed in 10% formalin and prepared for autoradiographic procedure according to conventional histologic technique. Grain counts were made of each slide.

The following observations and conclusions were made from this study:

1. The degradation product of insulin I^{125} was found to be deposited in a high distribution within the follicules of the thyroid as I^{125}. The maximum grain count was noted between 9-15 minutes and is therefore believed to be the maximum time for insulin degradation within the rat.
2. Accumulation of radioactivity was significantly higher in the thyroid tissue of all specimens than the other tissues studied.
3. There was a significant difference of grain counts between tissue specimens of all experimental rats.
4. No differences in the pattern of grain accumulation were found between the eight chemically induced diabetic and two non-diabetic experimental rats with the exception that the grain counts in the induced diabetic rats were consistently higher.
5. Grain accumulations were found within all the oral tissues evaluated with the exception of dentin.
6. The radioactivity within the dental pulp and bone was believed due to their proximity to vasculature.
7. Grain accumulation were noted within the intercellular substance of muscle, oral epithelium and crevicular epithelium. Within epithelium radioactivity was noted intercellularly primarily within the basal cell layer and stratum spinosum.
8. It is suggested that an increased dosage of insulin I^{125} may render a greater significance to the tissues studies.

CHAPTER VII

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APPENDIX

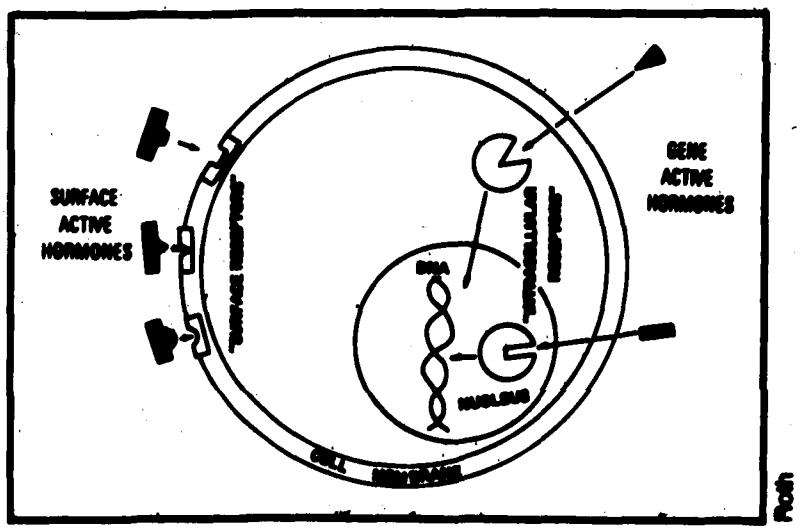

Fig. 1. Illustrates receptor site availability within the Plasma membrane of a cell.

(From Science News, Vol. III. No. 2, Page 17-32 Jan. 8, 1977.)

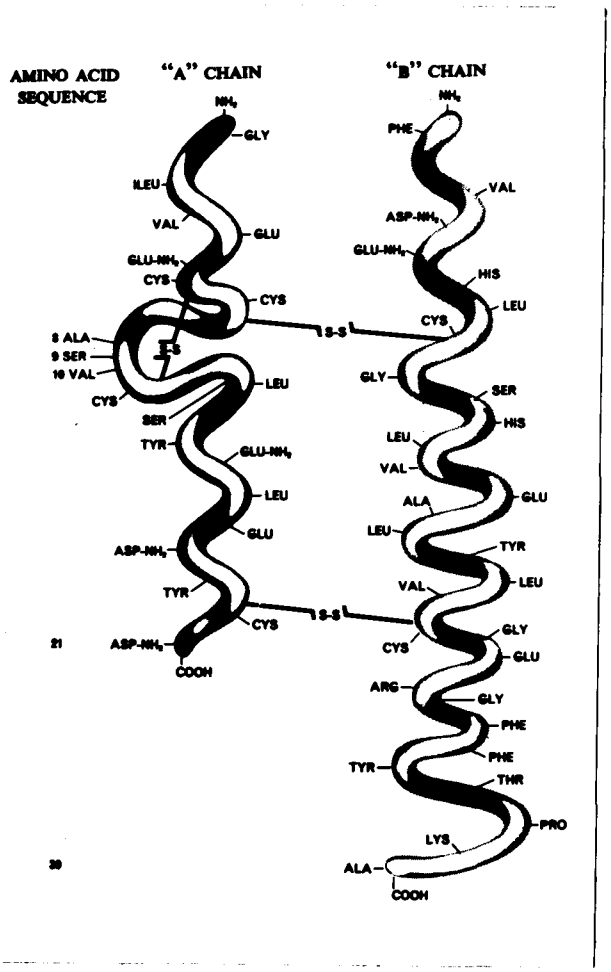

Fig. 2. Diagram of the beef insulin molecule, showing the sites of substitution between beef and human insulin.

(From Diabetes Mellitus by Waife, 7th Ed. 1976. Page 36.)

Fig. 3. Induced diabetic experimental rat.

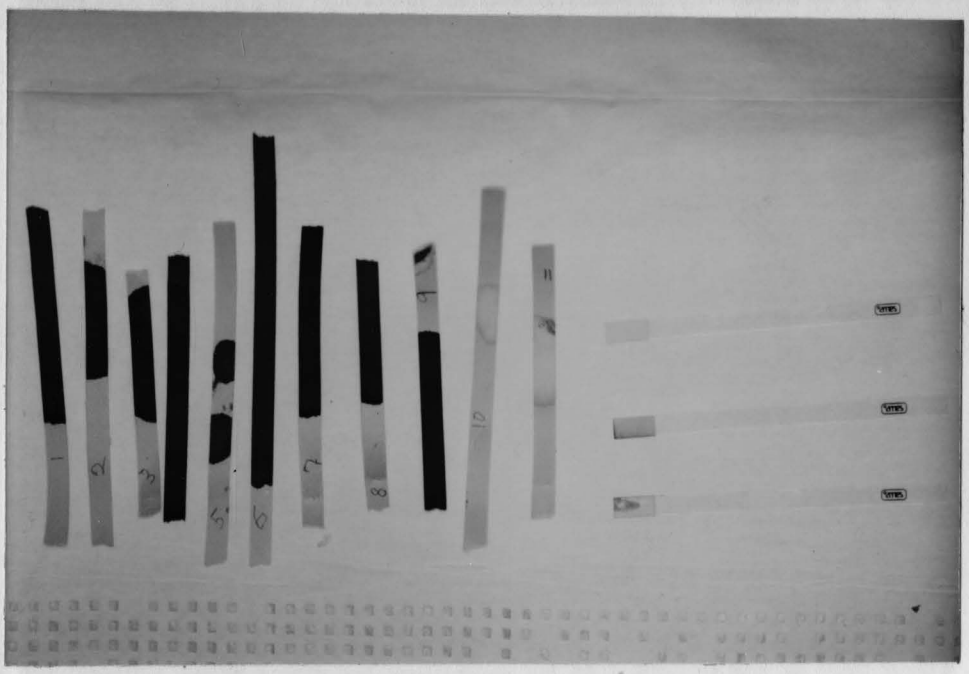

Fig. 4. Represents the Testape and Dextrostix values within control and experimental animals.

Fig. 5. Complete control (non-diabetic) experimental rat.

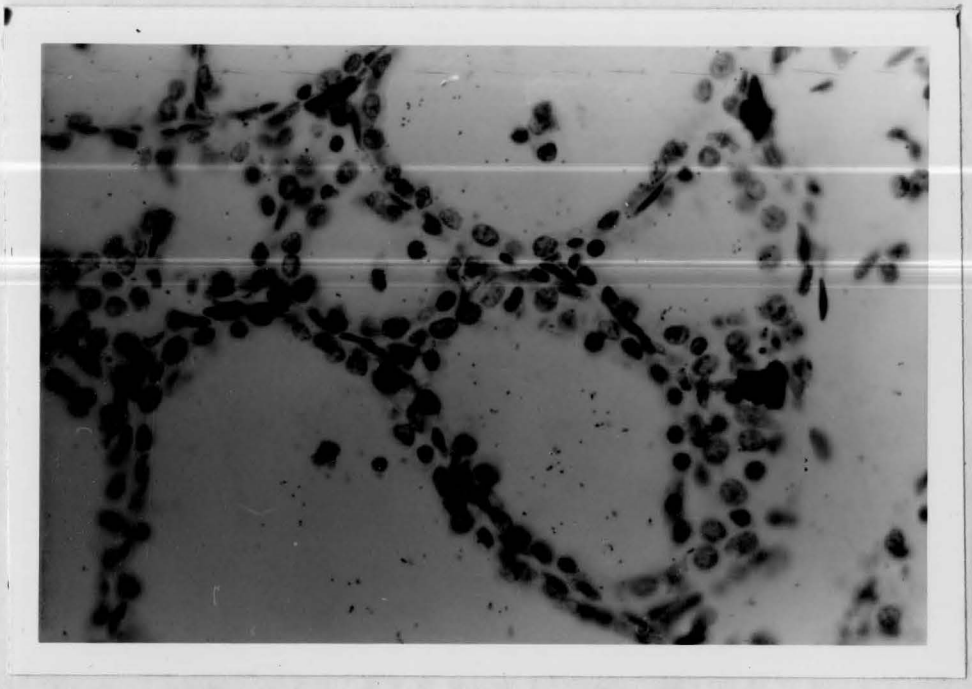

Fig. 6. Distribution of I^{125} in thyroid follicules after 5 minutes past injection of insulin I^{125}. (Original magnification 400X)

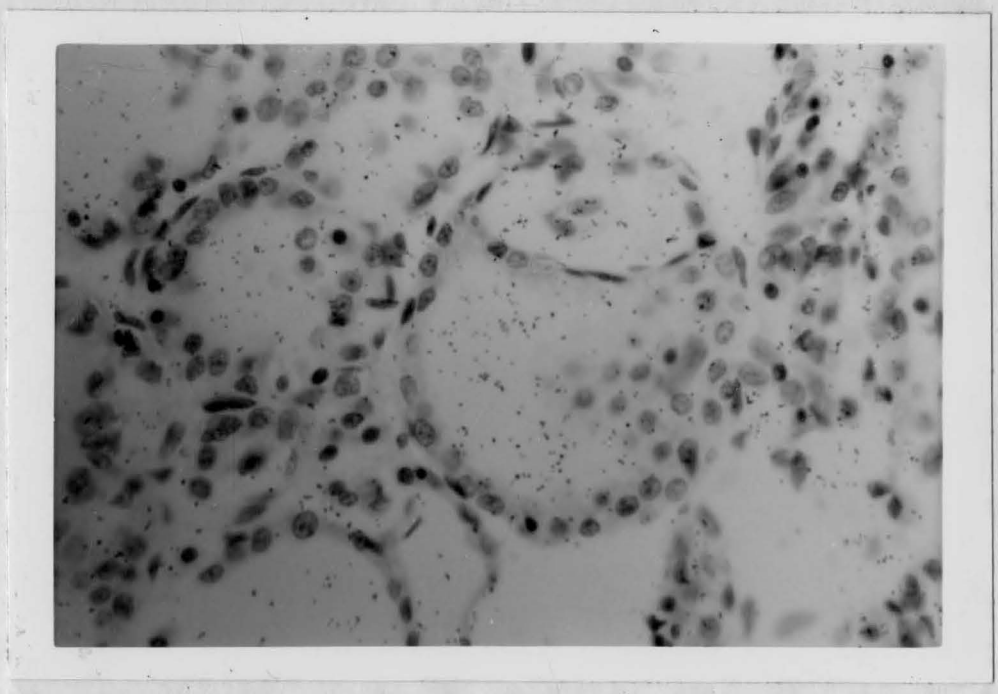

Fig. 7. Grain accumulations in thyroid follicules at 9 minutes past injection. (Original magnification 400X)

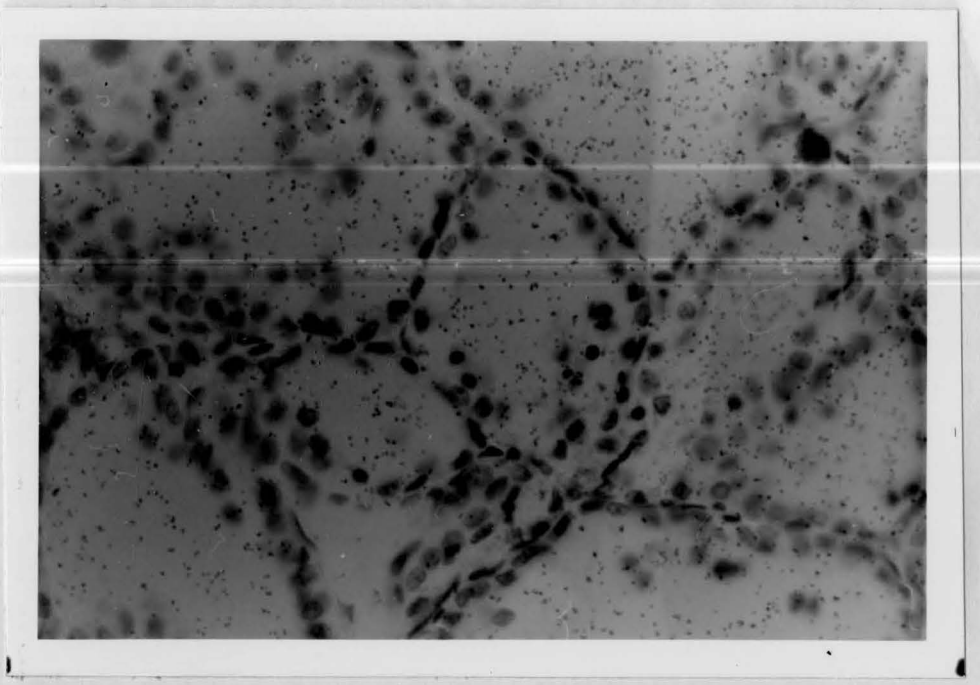

Fig. 8. I^{125} present in thyroid follicules at 12 minutes past injection. (Original magnification 400X)

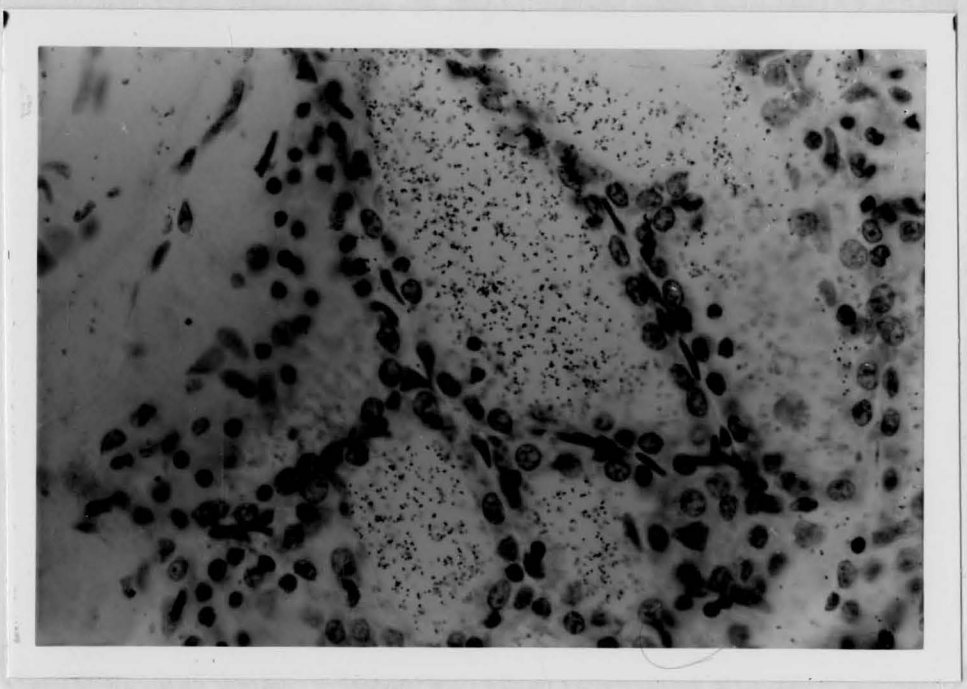

Fig. 9. Heavy accumulations of I^{125} are evident in the thyroid follicules at 15 minutes past injection of insulin I^{125}. (Original magnification 400X)

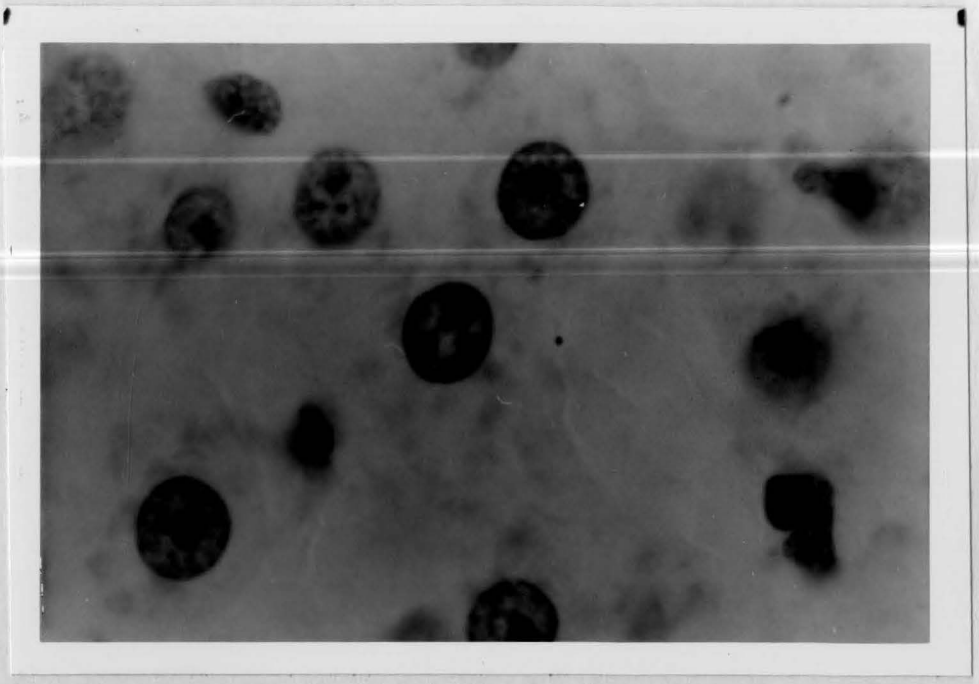

Fig. 10. Low grain distribution in the connective tissue of the liver at 5 minutes past injection of insulin I^{125}. (Original magnification 1000X)

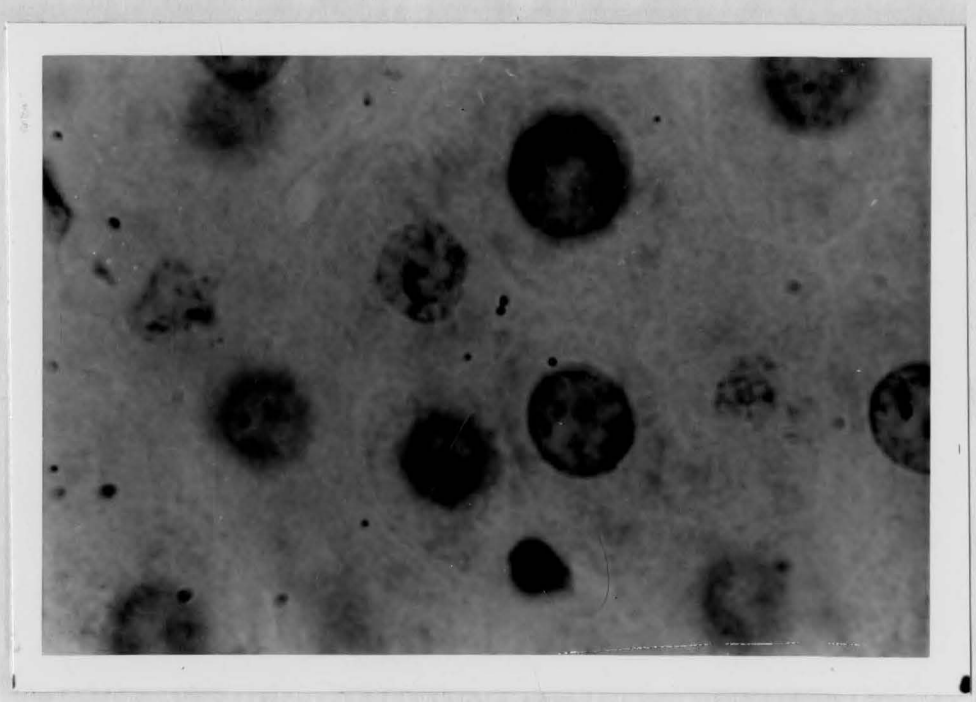

Fig. 11. Labelled insulin I^{125} in the connective tissue of the liver at 12 minutes sacrifice. (Original magnification 1000X)

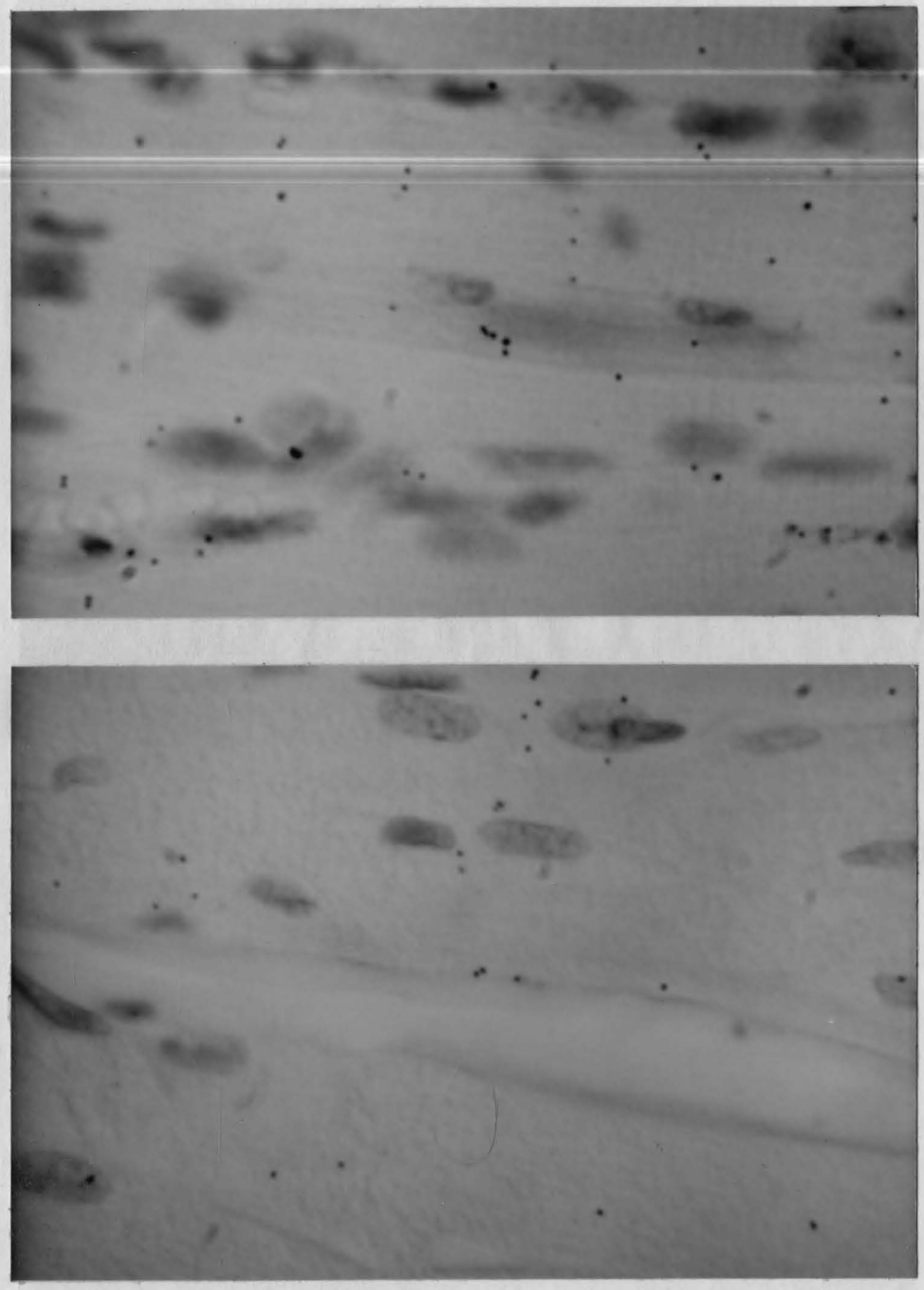

Fig. 12 & 13. Muscle 12 minutes sacrifice: note the close association of insulin I^{125} to the muscle Plasma membrane and intercellular substance. (Original magnification 1000X)

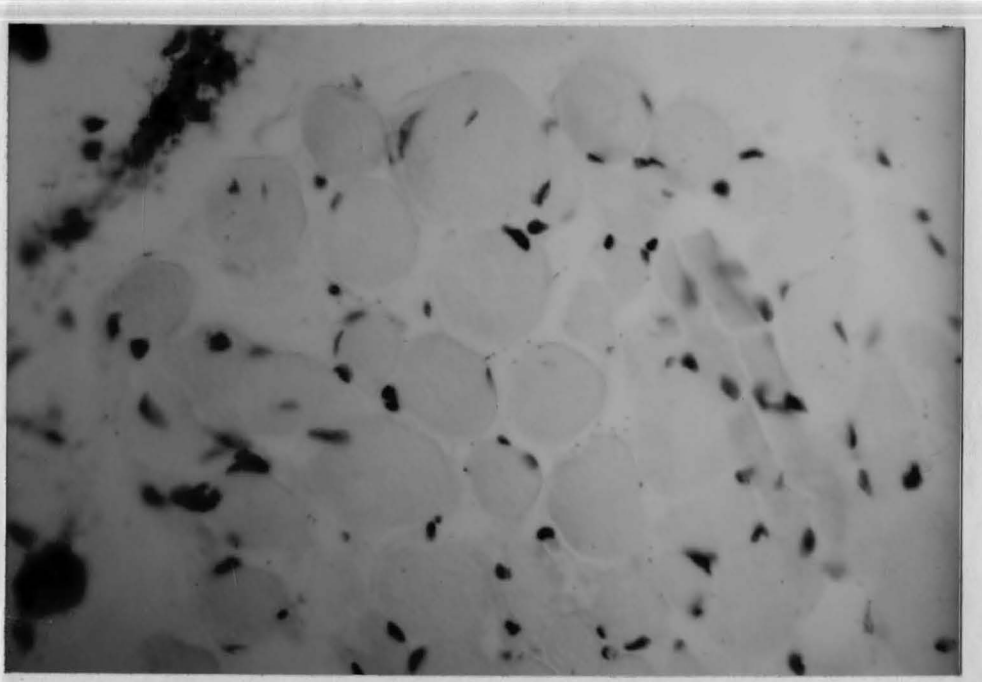

Fig. 14. Cross section of skeletal muscle at 12 minutes sacrifice: the grains are distributed throughout the intercellular substance. (Original magnification 400X)

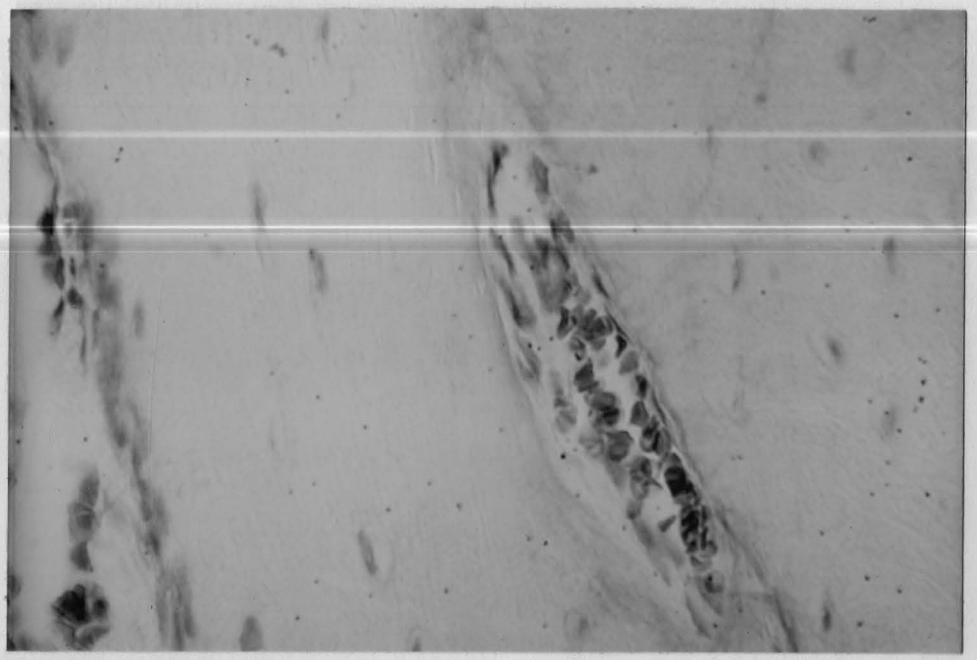

Fig. 15. Alveolar bone at 5 minutes sacrifice: grains are present within medulary bone in close relation to the vasculature. (Original magnification 400X)

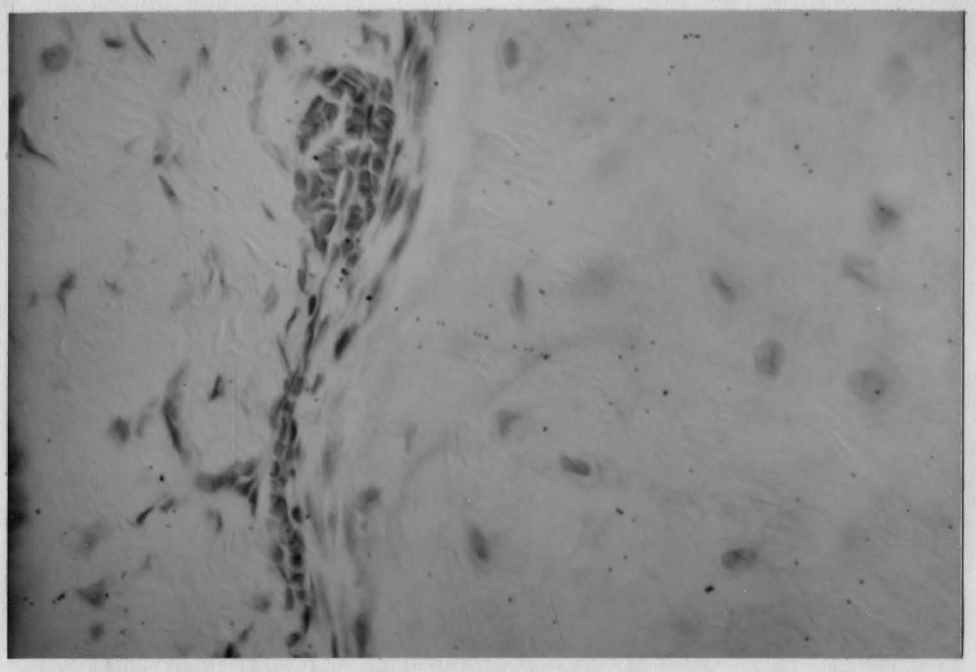

Fig. 16. Alveolar bone at 5 minutes sacrifice: labelled insulin I^{125} appears to be moving through a small blood vessel within the bone towards the periodontal ligament. (Original magnification 400X)

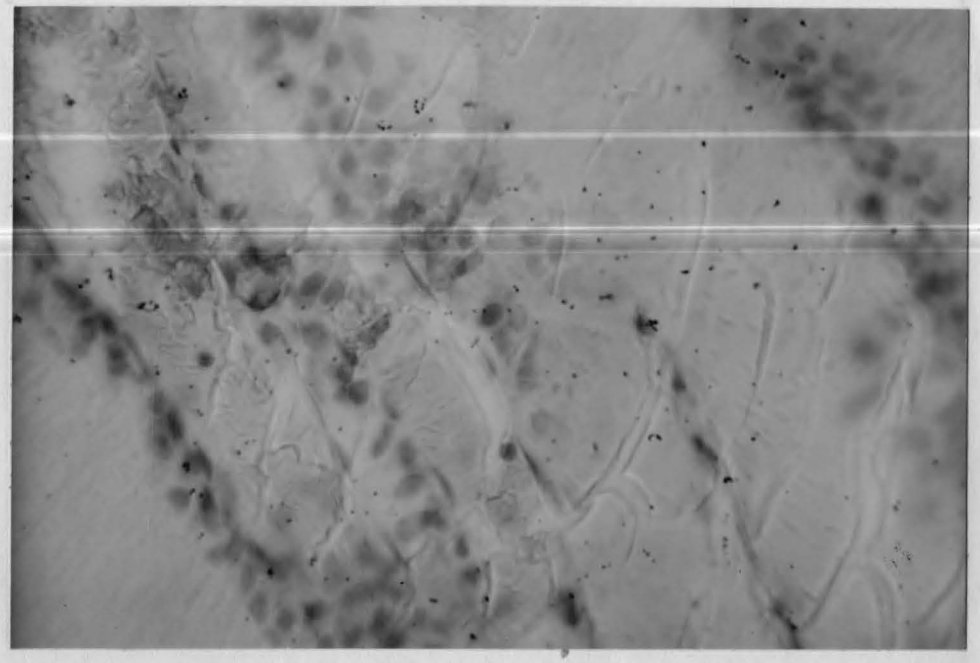

Fig. 17. Grains are distributed throughout the connective tissue of the pulp chamber at 12 minutes sacrifice. (Original magnification 400X)

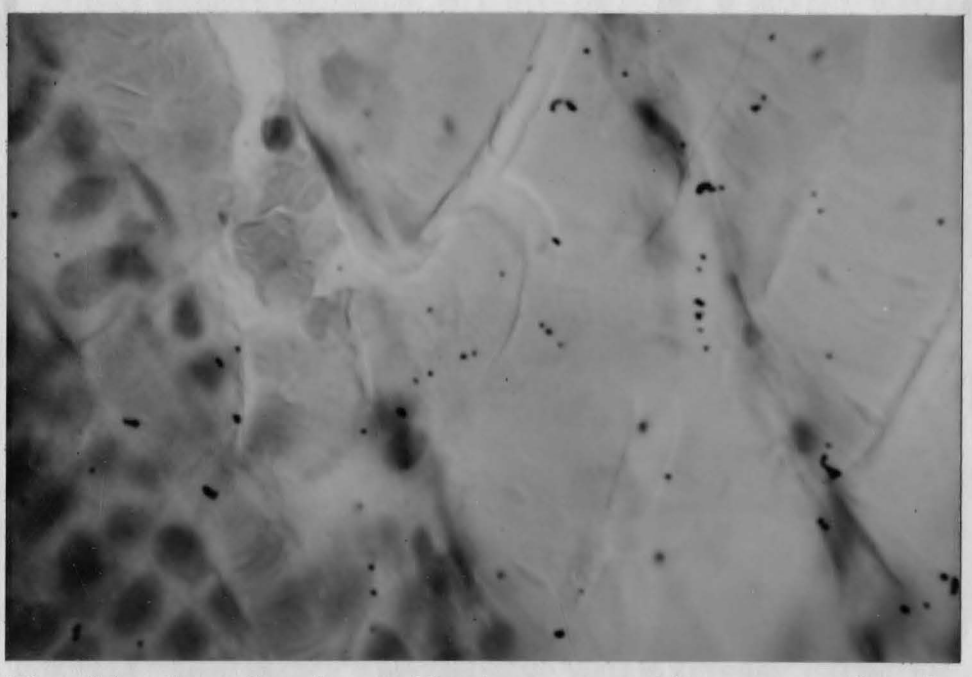

Fig. 18. Dental pulp at 12 minutes sacrifice: note the close proximity of the insulin I^{125} accumulations to the vasculature within pulp. (Original magnification 1000X)

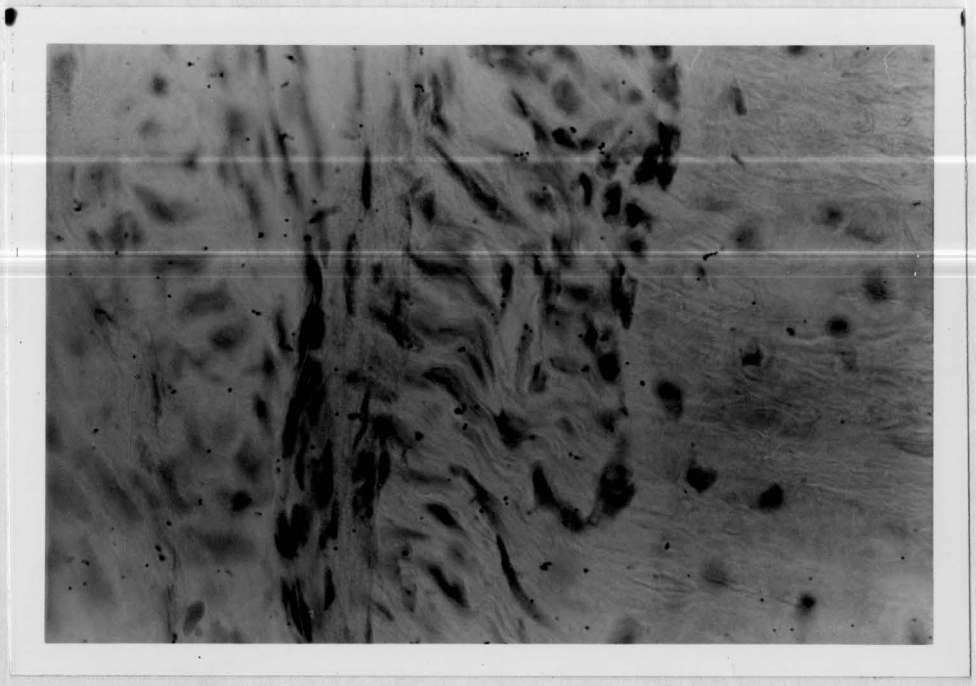

Fig. 19. Periodontal ligament at 12 minutes sacrifice: grains are evident within the connective tissue of the periodontal ligament and adjacent bone. Note decreased grains within the dentin. (Original magnification 400X)

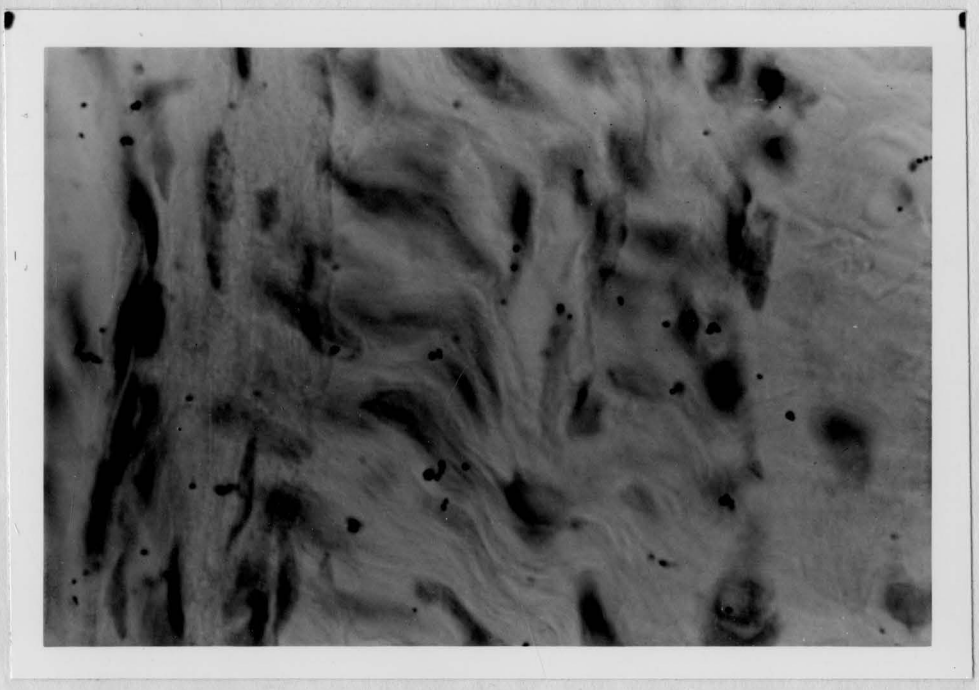

Fig. 20. Higher magnification of figure 19. (Original magnification 1000X)

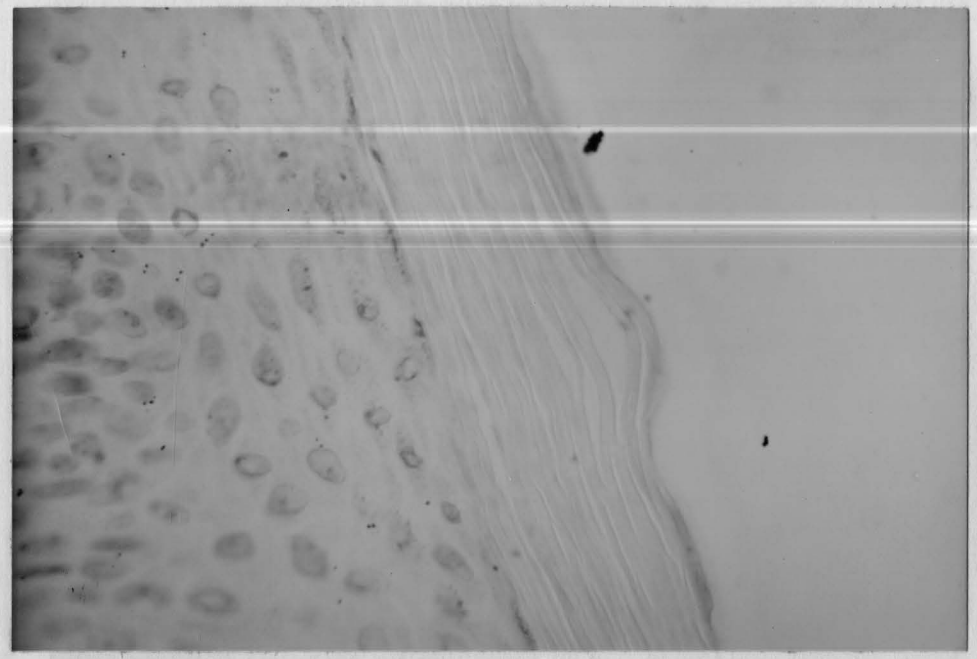

Fig. 21. Radioactive grains are present within the basal cell layer and stratum spinosum of the hard palate epithelium at 15 minutes sacrifice. (Original magnification 400X)

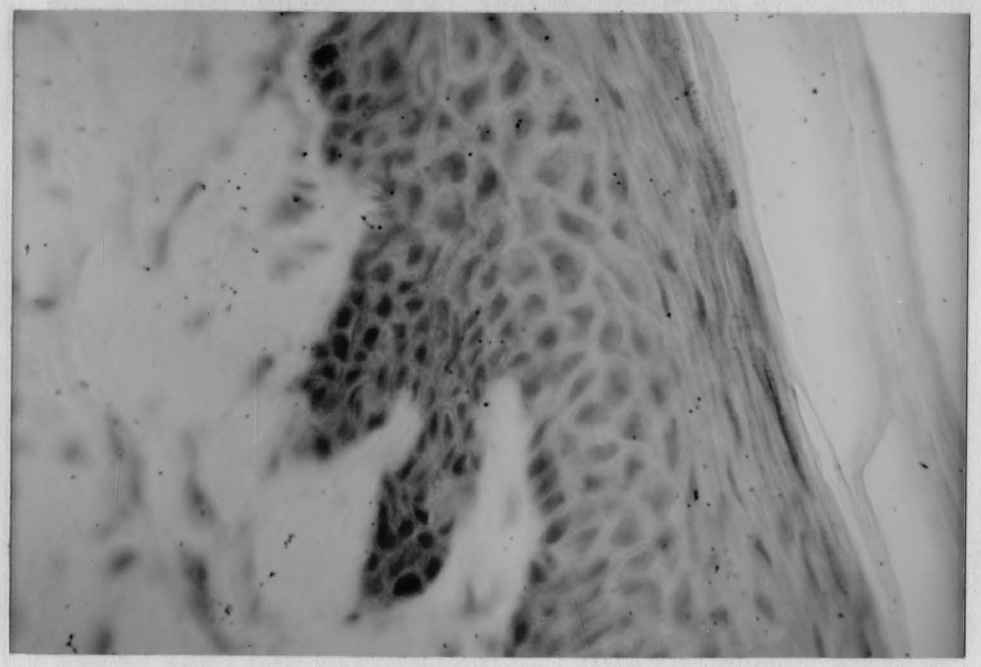

Fig. 22. Labelled insulin I^{125} is present within the dermis, basement membrane and the intercellular ground substance between adjacent epithelial cells of the gingivae at 15 minutes sacrifice. (Original magnification 400X)

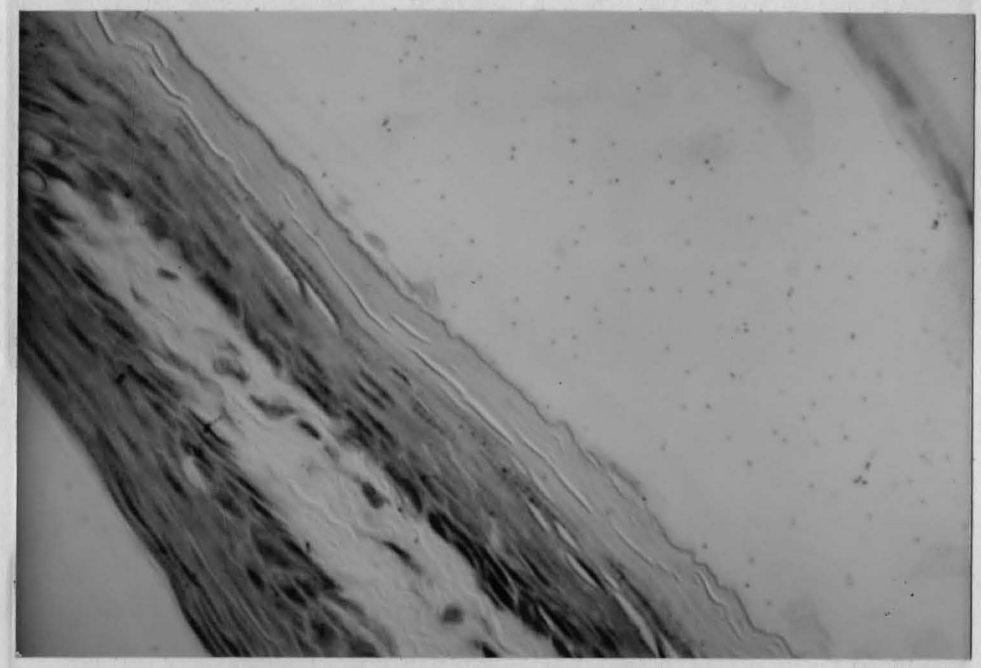

Fig. 23. Radioactive grains are distributed within the crevicular epithelium (left), and also heavy accumulations are noted within the gingival sulcus (right) at 9 minutes sacrifice. (Original magnification 400X)

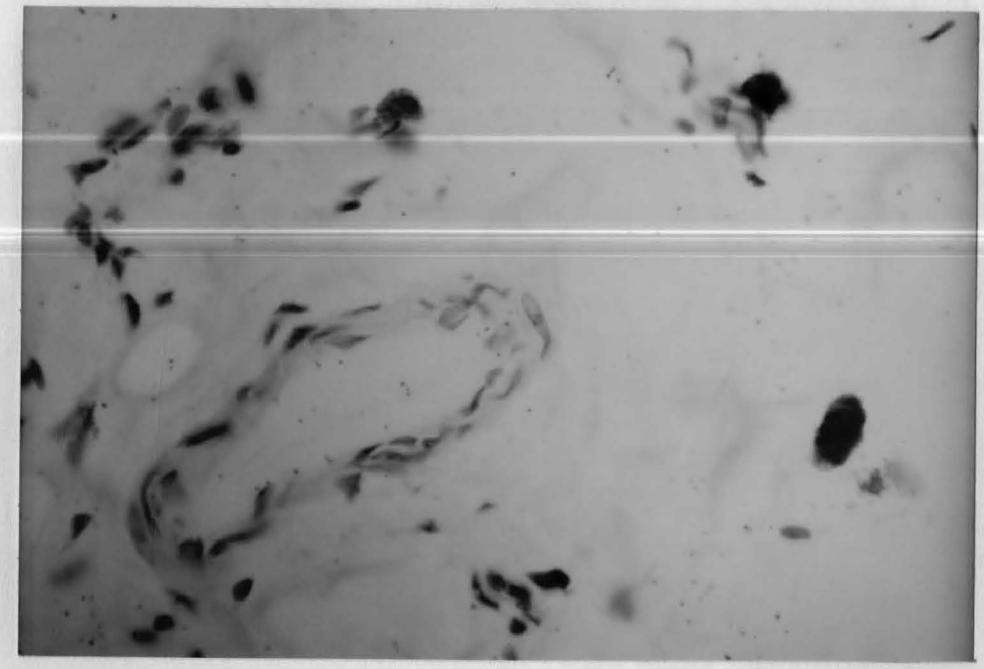

Fig 24. Insulin I^{125} is evident within the oral connective tissue at 15 minutes sacrifice. (Original magnification 400X)

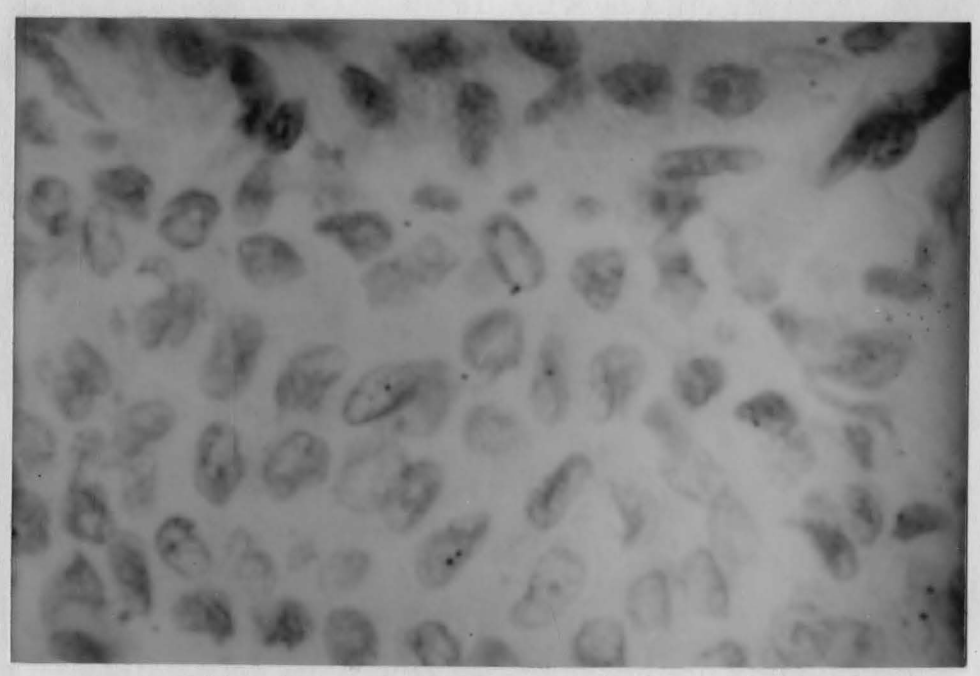

Fig 25. Submandibular gland at 15 minutes sacrifice: minimal grain accumulations are present within the glandular tissue. (Original magnification 1000X)

$$\text{tissue SS} = \frac{\Sigma(\Sigma Xm)^2}{Nm} - CT$$

Fig. 26. Formula = two way block analysis of variance for tissue SS.

$$\text{Subject SS} = \frac{\Sigma(\Sigma Xm)^2}{Nm} - CT$$

Fig. 27. Formula = two way block analysis of variance for subject SS.

$$K = K^* \sqrt{\frac{Ve}{Nm}}$$

Fig. 28. Formula = K distribution.

Rat No.	3rd day	4th day	5th day	6th day	7th day
Control #1 Non-diabetic	25 cc 10%	37.5 cc 15%	37.5 cc 15%	25 cc 10%	37.5 cc 15%
#2	137.5 cc 55%	100 cc 40%	150 cc 60%	150 cc 60%	175 cc 70%
#3	112.5 cc 45%	125 cc 50%	137.5 cc 55%	137.5 cc 55%	137.5 cc 55%
#4	125 cc 50%	150 cc 60%	150 cc 60%	175 cc 70%	150 cc 60%
#5	137.5 cc 55%	137.5 cc 55%	162.5 cc 65%	187.5 cc 75%	225 cc 90%
#6	125 cc 50%	150 cc 60%	150 cc 60%	200 cc 80%	250 cc 100%
#7	125 cc 50%	150 cc 60%	150 cc 60%	175 cc 70%	150 cc 60%
#8	125 cc 50%	125 cc 50%	150 cc 60%	125 cc 50%	125 cc 50%
#9	112.5 cc 45%	112.5 cc 45%	112.5 cc 45%	137.5 cc 55%	125 cc 50%
Control #10 Non-diabetic	37.5 cc 15%	37.5 cc 15%	50 cc 20%	25 cc 10%	25 cc 10%
Control #11 Non-diabetic	37.5 cc 15%	50 cc 20%	50 cc 20%	37.5 cc 15%	37.5 cc 15%

Table 1. Water consumption for diabetic and non-diabetic (control) rats, two days after injection of Streptozotocin. Percentage is calibrated according to a 250 cc volume of water replacement daily.

Rat No.	*Testape	**Dextrostix
Control #1 Non-diabetic	0	45%
#2	2%	250% or more
#3	2%	250% or more
#4	2%	250% or more
#5	2%	250% or more
#6	2%	250% or more
#7	1.5%	130%
#8	2%	250% or more
#9	1/5-2%	130%
Control #10 Non-diabetic	0	45%
Control #11 Non-diabetic	0	45%

Table 2. Table of Blood and Urine glucose level in experimental animals.

*Testape (Urine sugar semiquantitative analysis paper for in-vitro diagnostic).
By Lilly and Company, Eli; 307E McCarty St., Indianapolis, Ind., 46206.

**Dextrostix (Reagent strips for determination of blood - glucose levels qualitatively and quantitatively.)
By Ames Company, Division of Miles Laboratories, Inc. Elkhart, Indiana, 46514.

Animal No. Sacrifice time	thyroid	liver
Rat #6 Time = 5 minutes	15	19
Rat #5 Time = 9 minutes	135	32
Rat #4 Time = 12 minutes	185	37
Rat #3 Time = 15 minutes	355	25
Rat #2 Time = 28 minutes	17	8
Rat #7 Time = 4 hours	not readible	*
Rat #8 Time = 5 hours	269	*
Rat #9 Time = 7 hours	250	*
Rat #10 Time = 2 minutes Control (non-diabetic)	6	*
Rat #1 Time = 9 minutes Control (non-diabetic)	93	21

Table 3. Represents the average of two grain counts subtracted from their respective background counts at a magnification of X1000.

*Denotes animal in which the liver was not dissected.

Animal No. Sacrifice time	Dental Pulp	Periodontal ligament	Bone
Rat #6 Time = 5 minutes	25	22	32
Rat #5 Time = 9 minutes	64	22	20
Rat #4 Time = 12 minutes	29	26	17
Rat #3 Time = 15 minutes	23	32	16
Rat #2 Time = 28 minutes	29	30	14
Rat #1 Time = 9 minutes Control (non-diabetic)	51	17	12

Table 4. Represents the average of two grain counts subtracted from their respective background counts at a magnification of X1000.

Animal No. Sacrifice time	Crevicular Epithelium	Oral Epithelium	Skeletal Muscle	Oral Connective Tissue	Epithelial Attachment	Gland	Dentin
Rat #6 Time = 5 minutes	20	10	8	15	17	15	1
Rat #5 Time = 9 minutes	13	25	19	12	12	15	4
Rat #4 Time = 12 minutes	13	7	22	8	2	7	1
Rat #3 Time = 15 minutes	30	21	7	23	27	8	3
Rat #2 Time = 28 minutes	0	6	15	4	5	3	0
Rat #1 Time = 9 minutes Control (non-diabetic)	7	16	12	10	9	8	4

Table 5. Represents the average of two grain counts subtracted from their respective background counts at a magnification of X1000.

Tissue	$(\bar{x})$ mean of the tissue grain counts. High power X1000	Range
Thyroid	141.7	15 - 355
Dental Pulp	34.5	23 - 64
Periodontal Ligament	26.4	22 - 32
Liver	24.5	8 - 37
Bone	20.9	14 - 32
Crevicular Epithelium	15.4	0 - 30
Oral Epithelium	14.00	6 - 25
Skeletal Muscle	13.3	6 - 22
Oral Connective Tissue	12.6	4 - 23
Epithelial Attachment	12.6	2 - 27
Gland	9.9	3 - 15
Dentin	2.1	0 - 4

Table 6. Mean of the tissue grain counts computed through duration of experiment.

Variance Source	DF	SS	MS	F Value	F Table*
Subjects	4	9723.434	2430.85	1.444	2.59
Tissues	11	80744.384	7340.39	4.361	2.08
Interaction	44	74047.362	1682.89		
Total	59	164515.18			

Table 7. The results of the two way block analysis of variance are presented in this table.

Accept null hypothesis for the subjects (rats); there is no difference between subjects since the F value is less than the F table.

Reject null hypothesis for the tissues; there is a significant difference between tissues since the F value is greater than the F table.

* $\alpha = 0.05$

APPROVAL SHEET

The thesis submitted by Bahram Pourdeihimi
has been read and approved by the following committee:

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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 by the Committee with reference to content and form.

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

2/14/78
Date

Director's Signature