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An In Vivo Perfusion Apparatus for Studies on Gingival Disease in the Albino Rat : Assessment by a Perfusion of the Enzymes Collagenase and Hyaluronidase

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AN IN VIVO PERFUSION APPARATUS FOR STUDIES ON GINGIVAL DISEASE IN THE ALBINO RAT: ASSESSMENT BY A PERFUSION OF THE ENZYMES COLLAGENASE AND HYALURONIDASE

By

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CHAPTER I

AN IN VIVO PERFUSION APPARATUS FOR STUDIES ON GINGIVAL DISEASE IN THE ALBINO RAT: ASSESSMENT BY A PERFUSION OF THE ENZYMES COLLAGENASE AND HYALURONIDASE

INTRODUCTION

There is mounting evidence that indigenous microorganisms of the dental plaque and gingival sulcus have the potential to elicit or modify the inflammatory response characteristic of gingival disease (1-7). In a susceptible host the manifestation of this potential seems to depend both on an increase in the number of localized microorganisms and on a shift in the balance of flora (7-11). Some investigators have reported microorganisms within the gingival tissues (12-22). Others have seen no evidence for bacterial invasion of the gingivae (23-28) except as a result of trauma, (29, 30) and there is no evidence for a change in virulence (10, 31, 32).

The normal flora of the plaque and gingival sulcus has the capacity to produce and release such biologically active substances as hydrolytic enzymes, associated antigens, endotoxins and various other metabolites (2, 6, 23, 27, 28, 33-50). Under appropriate conditions of microbial growth and host susceptibility, microbial agents induce initial cell injury in the crevicular epithelium or in the subjacent connective tissue upon penetration of the epithelium. The initial cell injury in turn releases intracellular chemicals which mediate and govern the process of inflammation in the host tissues (6, 51-54). Prolongation of the inflammatory response in the periodontium leads to a

breakdown of the periodontal fibers, apical migration of the epithelial attachment and alveolar bone resorption typical of periodontal disease (6).

Thus the broad features of the interaction between the oral microbiota and the host tissues are clear. It is the specific mechanisms by which these organisms change the environment in the sulcus to initiate inflammation of the gingival tissues that are not firmly established. There has long been a need to develop an experimental model of the pathogenetic situation which will deliver local factors associated with the oral microbiota to the host tissues in such a manner that reasonable inferences might be made as to their pathogenetic role in periodontal disease.

It was therefore decided to develop an in vivo perfusion apparatus capable of continuously delivering regulated volumes of prepared solutions to localized, contralateral, intraoral areas in rats without extraneous tissue trauma or unnatural restraint upon the animals. The enzymes hyaluronidase and collagenase, already known to be elaborated by members of the oral flora and previously implicated by other investigators of the pathogenesis of gingival and periodontal disease (6, 27, 28, 36, 55-61) were employed to complete the experimental model and to provide a means of assessing the in vivo perfusion apparatus.

CHAPTER II

REVIEW OF THE LITERATURE

Gingival debris is composed almost entirely of bacteria (8, 10). Of particular interest in this investigation are the groups of crevicular organisms which elaborate the enzymes collagenase and hyaluronidase.

More than 68% of the microorganisms have been shown to produce the enzyme hyaluronidase (62). These active organisms are the alpha hemolytic streptococci together with strains of staphylococci and certain of the diphtheroids (34, 40, 62-64). The enzyme hyaluronidase has itself been detected both in the gingival sulcus and in saliva (36, 58, 61, 63, 65). Salivary levels of the enzyme in young adults were found to be relatively constant as well as reproducible within a given range of activity for that individual (61, 63, 65, 66, 67). Gibbons, however, found no difference in hyaluronidase activity of gingival debris between normal and periodontally involved individuals (10, 32). Apparently the increase in hyaluronidase activity of saliva is a reflection of a greater mass of debris in individuals with periodontal disturbance (10, 32).

Hyaluronic acid and chondroitin sulfate A and C, the mucopolysaccharide substrates of hyaluronidase are found in connective tissues (68-71). They are also thought to contribute to the adhesion between epithelial cells and between these cells and hard surfaces (3, 56, 72-76).

In connective tissue the action of the enzyme gives rise to increased tissue permeability (6, 77, 78). Thilander has explored the effect of hyaluronidase on epithelium in the gingival sulcus (3, 56). Using the electron microscope, he demonstrated a widening of the intercellular spaces of human gingival epithelium after local application of this enzyme (3). Murphy and Stallard demonstrated the penetration of Trypan blue between epithelial cells after topical application of hyaluronidase to the gingival sulcus of the squirrel monkey (78). Disturbance of the intercellular ground substance may increase the permeability of crevicular epithelium and facilitate the entrance of enzymes such as collagenase through the gingival crevice.

A true collagenase enzyme capable of hydrolyzing native collagen is elaborated by only a few oral microorganisms. One of these organisms is known as Bacteroides melaninogenicus. B. melaninogenicus is a small Gram-negative, anaerobic, nonsporulating rod which produces a black pigment (79). Though very few preschool children harbor this organism in their mouths, the prevalence of this microorganism shows a sharp increase in the period of mixed dentition (80, 81). By the time of adolescence B. melaninogenicus is almost universally present in the normal oral flora (8, 80, 82-84).

B. melaninogenicus has been regarded as an opportunist (85, 86). Under appropriate conditions it was thought to be an important pathogen in mixed anaerobic infections of mucous membranes produced by oral microorganisms (86-88). The number of B. melaninogenicus are strikingly increased in periodontal disease (8, 9, 59, 89). Indeed Courant and Bader utilizing a special tissue sectioning technique showed the organism in biopsied connective tissue from inflamed human gingivae (22).

Studies of thirty-one strains of B. melaninogenicus revealed that all were proteolytic (90, 91). Production of collagenase capable of hydrolyzing native collagen (38, 41) is maximal after 48-72 hours' incubation in microbial culture (92). Apparently the enzyme is not secreted but released from the cells when they undergo lysis (38, 41). This unusual pattern of collagenase formation may be related to the chronic nature of periodontal disease (38, 41).

The enzyme is stable in solution at 4°C. for at least one month and indefinitely when frozen. It functions best over a pH range of 6.8 to 7.3 (38) with an optimum pH of 7.0 in 0.05 M Tris buffer. Calcium ions are required for adsorption of the enzyme to collagen while another unknown metal ion also appears to be essential for activity (38, 93).

Collagenase has an unusually narrow specificity and hydrolyzes the collagen protein itself rather than the mucoprotein moiety in intimate contact with the collagen (94). In addition, however, collagenase has been shown to inhibit the development of fibroblasts (95) and to disrupt leukocytic lysosomes (96). There is recent evidence that lysosomes and other endogenous cell fractions themselves produce and release a collagenase (97-102). Thus it is possible that destruction of gingival collagen may begin with bacterial collagenase originating in the gingival sulcus (36, 57, 58) and continue with endogenous collagenase released in response to an inflammatory process (103, 104).

Initial penetration of the epithelial tissue by bacterial collagenase may occur through enzymatic hydrolysis of glycoproteins in the intercellular substance. It is already known that proteolytic enzymes such as trypsin diminish the adherence of epithelial cells to hard

surfaces and to each other by digesting the glycoproteins of the cell surface (105). Using repeated injections of collagenase into the skin, Klingsberg and Butcher explored the effect of the bacterial enzyme on the collagen which forms a major part of fibrous connective tissue (106). They found that collagenase was relatively ineffective in promoting connective tissue lysis while papain and trypsin were highly lytic. In addition Thonard has reported that multiple doses of the enzyme failed to produce measurable tissue changes apparently indicating rapid clearance of the enzymes from the tissues (107). Animals with a prolonged challenge did show histopathologic changes similar to periodontitis. These changes were suppressed when the immunosuppressive drug Imuran kept the immunological activity at a minimal level. Again it seems clear that the susceptibility or the responsiveness of the host is involved in the tissue breakdown observed in the presence of collagenase.

In summary there is clear evidence of the presence of both hyaluronidase and collagenase in the gingival sulcus and of the appropriate substrates of these enzymes in the gingival tissues. There is evidence, too, that hyaluronidase is capable of penetrating the interepithelial cell spaces as well as hydrolyzing the hyaluronic acid of the connective tissue ground substance. It has been thought that the penetration and subsequent action of collagenase would thus be facilitated. However, the experimental evidence for the pathogenetic role of hyaluronidase and collagenase in gingival disease is uncertain. The study of DeVincenzo and Jeffries alone has demonstrated pronounced inflammatory potential of collagenase and hyaluronidase when they were

delivered together into the subjacent connective tissues (108). Despite this evidence there has been difficulty assessing the role of these substances in the pathogenesis of periodontal disease with a suitable experimental model.

The development of a suitable experimental model has been hampered by the lack of a continuous, atraumatic means of delivering solutions to the tissues. Most studies have employed either the periodic, topical application of agents to the surface of the gingivae (3, 56, 78) or the intermittent injection of the solutions into the gingival tissues (55, 106, 107). Both failed to maintain a continuous application of the solutions. This would have been distinctly desirable since human dental plaque and its breakdown products are often continuously rather than intermittently present.

The experimental model of DeVincenzo and Jeffries sustained the continuous application of the solutions, but required the trauma of injection (108). The interaction between trauma and the exogenous factors complicated evaluation of tissue changes. Furthermore in both situations the animals were subjected to the stress of repeated physical restraint. It was therefore proposed to develop an experimental situation which would insure the continuous, regulated delivery of test solutions to localized areas intraorally without the trauma of injection nor the necessity of physical restraint.

CHAPTER III

METHODS AND MATERIALS

Subjects

Twelve male Sprague-Dawley derived rats (Holtzman Company, Madison, Wisconsin) weighing from 322 to 375 grams were previously fed Purina Chow pellets and water ad libitum. During the experimental period, however, they were fasted to minimize masticatory trauma to the intraoral apparatus.

Enzymes

The powdered enzymes were obtained from the Sigma Chemical Company. The hyaluronidase (bovine testes) had an activity of 300 N. F. units/mg. The collagenase (Cl. histolyticum) had an activity of 120-200 units/mg. They were stored at 4°C. to prevent inactivation which may occur at room temperature.

Solutions

The following solutions were employed in the conduct of the study:

Enzyme solutions

0.5%, 1% and 2% hyaluronidase

0.5%, 1% and 2% collagenase

Control solutions

tap water

double de-ionized water

0.05 Molar Tris buffer (2-amino-2 hydroxy-methyl-1 3 propandiol) pH 7.5

Disclosing solution

2% Trypan blue

The hyaluronidase was dissolved in double de-ionized water. The collagenase was dissolved in 0.05 M Tris buffer. The fresh solutions were prepared every six hours, stored within the perfusion syringes and maintained at 4-7°C.

Apparatus

The experimental apparatus consisted of an infusion pump connected by means of mounted plastic tubing to a fixed intraoral perfusion needle. The 25 gauge needle was modified so that its beveled end was adapted to the mesial buccal surface of the maxillary first molar of a rat. With this arrangement, experimental solutions could be delivered to the mesial sulcular area. At the mesial buccal line angle of the maxillary third molar the unbeveled end of the needle was curved at a 45° angle to the buccal surface. The 45° angle bend was necessary to accommodate the rat's mandibular movement.

Polyethylene tubing (PE 20, Clay Adams, New York) with an internal diameter of 0.018 inches was placed over the unbeveled end of the needle to the distal surface of the first molar. Orthodontic ligature wire (0.012) was tightly wrapped six times around the tubing and needle in the area adjacent to the buccal surface of the second molar (Figure 1).

A 20 gauge trocar was passed subcutaneously from the buccal mucosal surface opposite the third molar (Figure 2) and directly posterolaterally so that it would emerge at the back of the neck (Figure 3).

The trocar was removed after the free end of the tubing was passed through. The tubing was drawn posteriorly until the beveled end of the needle approximated the mesial surface of the first molar. The unwrapped ends of the ligature wire were passed under the mesial and distal contacts of the second molar. The ligature wire was secured by twisting the ends together on the palatal surface. The twisted end was trimmed to extend five millimeters from the interproximal area and bent toward the first molar to keep the animal's tongue away from the area of perfusion (Figure 4).

The diagrammatic representations of the apparatus (Figures 5, 6) show the needle and the tubing tightly secured so that the beveled surface closely approximates but does not touch the mesial sulcus. This same procedure was performed on the contralateral side. To prevent displacement of the tubing, it was passed through a plastic pack worn by the animal (Figures 7, 8) and was secured to a vertical metal rod on the pack by tape before connecting it to the perfusion pump. The plastic pack itself was mounted and held on each animal by means of a plastic covered spring harness (Figure 9). Adjustment of the harness to each animal was facilitated by wire loops on one side of the pack, through which the spring was passed. The desired tension on the spring harness was maintained by a small rubber disk.

To provide more protection for the portion of the perfusion tubing within the cage, a closed-coil spring was constructed and attached to the vertical metal rod on the pack. The spring prevented the rat from chewing the tubing, but allowed reasonable freedom of movement. The upper end of the spring was attached to a horizontal rod supported on a

ring stand. The pack and harness were suspended at a height acceptable to accommodate the rat's movements. The polyethylene tubing was passed up through the spring and a simple pulley suspension was arranged to provide the appropriate tension on the tubing at all times.

Freely rotating circular cages 9 inches in diameter and 16 inches in height were constructed to house the rats individually and aid in unwinding the tubing caused by the animal's circular movements. The rats were otherwise unrestrained and allowed to move in any direction within the confines of the circular cage (Figure 10). Finally the free end of the tubing was attached to a compact infusion pump (Model #975, Harvard Apparatus Company, Mills, Massachusetts) capable of delivering solutions at the rate of 0.65 ml/day to 1,152.0 ml/day (Figure 11).

Procedure

To facilitate placement of the perfusion apparatus, the animals were anesthetized by intraperiotoneal injection with pentobarbital sodium according to body weight (50 mg./Kg) and then placed on a rat board. Each syringe was filled with fresh solution and firmly positioned in the perfusion pump. A temperature range of 4-7°C. was maintained by the use of a "dry ice pillow" adapted to the upper aspect of the syringes and stabilized with tape. The polyethylene delivery tubing was cut to 36 inches to minimize the time for the solutions to pass from the pump to the perfusion needle and thus to reduce the exposure of the enzymes to room temperature.

The rate of perfusion was also adjusted to minimize inactivation of the enzymes in the apparatus and, at the same time, to assure

localization at the perfusion site. A delivery rate of 4.8 ml/day was established in the initial series of animals. In later series it was found that this could be increased to as much as 7.1 ml/day without complicating the localization of the delivered solution.

The time of perfusion was established at four days. In animals number 4 through 9, the fresh enzyme solutions were prepared every 12 hours. Animals number 10 through 12 received fresh enzyme preparations every six hours. This was the limit of convenience in sustaining the activity of the enzyme solutions and allowed sufficient time to elicit histological changes.

When two enzymes were employed, the solutions were perfused in sequence. In every instance but one, hyaluronidase was perfused first, followed by the collagenase solution. The rationale was the reported capability of hyaluronidase to penetrate epithelium and then hydrolize the hyaluronic acid of the connective tissue ground substance thus facilitating the entrance and subsequent action of collagenase.

After perfusing the animals with test solutions on the experimental side and with the appropriate control solution on the contralateral side, all the animals were perfused on both sides with 2% Trypan blue to determine localization of the delivered solution and to observe possible dye penetration in the crevicular epithelium. In the experimental series dye perfusion was conducted for two hours at the same rate as the prior perfusion of test solutions.

At the termination of all perfusions, needle placement was checked to see that a relatively constant position was maintained. The animals were sacrificed with an overdose of pentobarbital sodium. They were then

weighed, secured to the rat board and the palates dissected free. The palates were placed in 10% formalin for 24 hours. Demineralization was accomplished by placing the palates in Rapid Bone Decalcifier (RBD) from DuPage Kinetic Laboratories for eight hours. Finally, the palates were embedded in paraffin.

Histologic sections six microns in thickness were taken from the lingual aspect of the specimen advancing through to the buccal aspect of the maxillary molars. One section every 100 microns was preserved for examination until the center of the tooth was reached. At this point, a section was preserved for every 50 microns cut in order to accurately record tissue variations resulting from enzyme perfusion. This procedure was followed to a point two sections beyond the buccal aspect of the maxillary molars. A total of 15 to 18 sections were removed for examination from each experimental site. The sections were mounted and examined for localization and penetration of Trypan blue. The sections were then stained with hematoxylin and eosin and studied microscopically at 63x to 400x magnification.

Evaluation

Localization of the perfusion fluids was evaluated by gross examination for the distribution of Trypan blue staining and the pattern of inflammation when present. Penetration of the crevicular epithelium by enzyme action was assumed from the evidence of inflammatory response in the epithelium and subjacent tissues. The capacity of hyaluronidase and collagenase to elicit or modify an inflammatory response was deduced from the histological evidence of inflammatory changes.

Observations were directed toward the presence and type of inflammation and exudate adjacent to the mesial gingival crevice. Particular assessment was made separately of the crevicular epithelium and the subepithelial connective tissues. This was facilitated by a lens which projected a square outline in the center of the microscopic field 200 microns by 200 microns at 400x magnification. The neutrophils and changes in cellular detail were observed. Possible changes in cellular details include acantholysis, karyorrhexis and karyolysis. Notice was also taken of intracellular edema and microabscesses.

The subepithelial connective tissue was inspected for neutrophils, vascular congestion and visible evidence of edema.

Tissue damage to the mesial sulcular area induced by the needle and ligature wire was determined by comparing the histologic sections taken from the control side with the experimental side.

CHAPTER IV

RESULTS

Observations were made in 12 experimental animals (Table I).

Control Animals

Three animals were selected as controls to observe localization of the solution and any histological change the presence of the needle and ligature wire might induce on the mesial sulcular area of the maxillary first molar. These animals were perfused on one side with tap water at the rate of 4.8 ml/day. The contralateral side was left undisturbed.

Prior to sacrifice of an animal at the end of one day, four days or seven days, each animal was perfused for five hours with Trypan blue. While perfusion continued the animals were anesthetized and the drops of Trypan blue solution emerging from the needle were observed for several minutes. The drops touched the mesial sulcular area and then were dispersed up the palatal and buccal line angles of the rat's maxillary first molar. In each of these three control animals, the perfused dye was localized to the mesial sulcular area of the maxillary first molar (Figure 12).

Microscopic examination of control sections revealed that the dye did not penetrate the gingival crevice on either side, yet there was slight staining of the keratin near the margin of the crevicular epithelium. Further histological inspection revealed a mild inflammation with little difference between the perfused and undisturbed sides in the area under observation.

The crevicular epithelium was comprised of stratified squamous keratinized epithelium, 3-12 rows thick, becoming narrower at its base adjacent to the cemento-enamel junction. A stratum corneum was present to approximately half the distance from the marginal area to the cemento-enamel junction. A prominent stratum granulosum with keratohyalin granules was found below the stratum corneum. The stratum spinosum was several cells in width and the cells had a polyhedral shape. The stratum basale was present with a relatively normal configuration. Rete ridge formation was seen on both sides. No changes in cellular detail were observed. No significant difference in the number of neutrophils between the perfused and undisturbed side could be found.

The subepithelial connective tissue of both the perfused and unperfused side, demonstrated cells, fibers, vessels and ground substance. The cells were primarily fibroblasts, a few neutrophils, fewer lymphocytes. Occasional mast cells, plasma cells and macrophages were seen. A small number of vessels were observed with walls composed of thin platelike endothelial cells. Most of this area of connective tissue was comprised of strands of wavy pink-staining collagen fibers. Wider spacing between the cells and structures within the tissue, presumptive of edema, was observed on several sections of the animal perfused for seven days.

Experimental Animals

The remaining nine animals were used for the experimental perfusion of the selected enzyme solutions with terminal perfusion of Trypan blue for two hours--which was found to be sufficient time for staining.

Two of the animals were perfused on one side with hyaluronidase in solution and on the contralateral side with double deionized water. In one animal (Table I, #5) a 1% solution of hyaluronidase was delivered at the rate of 4.8 ml/day for four days. In the other animal (#11) a 2% solution of the enzyme was delivered at 7.1 ml/day for four days.

Five additional animals were perfused sequentially in one side with both hyaluronidase and collagenase enzyme solutions. In each case the control side was perfused with both double deionized water and with Tris buffer. In one animal (#6) a 0.5% solution of each enzyme was delivered for two days at the rate of 4.8 ml/day. In two animals (#7, 9) a 1% solution of each enzyme was delivered for a day and a half at 5.4 ml/day. In one animal (#8) a 1% solution of each enzyme was delivered for two and one-half days at 5.4 ml/day. And finally in one animal (#12) a 2% solution of each enzyme was delivered for two days at 7.1 ml/day. Examination of the slides for Trypan blue revealed that the dye had not actually penetrated the crevicular epithelium in any of the animals. Intracellular and intercellular staining in the crevicular epithelium with Trypan blue was not discernible to a significant extent in any of the experimental or control sections (Figure 13).

A histologic evaluation of the seven animals perfused with hyaluronidase or hyaluronidase and collagenase demonstrated no discernible difference between the control and experimental areas. There were also no remarkable differences among the animals subjected to the different experimental situations. Furthermore, comparison between these animals and those of the original control group revealed no significant differences.

Rather the typical histological picture of the marginal gingivae revealed a normal pattern of keratin with an increase in the granular layer projecting into the sulcus. The attachment area showed continuity in the basal cell layer thus indicating an intact epithelium. Monocytic infiltration accompanied by a few other leucocytes immediately under the epithelial layer was observed. Scant mitotic activity was noted in the connective tissue. There was an increase in vascularity (Figures 14-21).

Finally two animals were perfused on one side with collagenase in solution and on the contralateral side with Tris buffer. In one animal (#4) a solution of 1% collagenase was delivered at the rate of 4.8 ml/day. In the other animal (#10) a 2% solution of the enzyme was delivered at 7.1 ml/day. The animal perfused with the 2% solution presented extensive histologic inflammatory changes in both experimental (Figures 22, 23) and control (Figures 24, 25) areas. There was somewhat lesser involvement of the control side.

Changes in epithelial cellular detail (#10) were not discernible on an individual basis because of the extreme polymorphonuclear leukocytic cellular infiltration. There was a generalized loss of keratin along with the desquamation of the epithelium on both sides of the free gingiva. A relatively dense inflammatory infiltrate accompanied by disruption of the enamel cuticle was observed in this area. There was a fibrinous exudate with tremendous numbers of neutrophils adjacent and coronal to the crevicular epithelium. Further evidence of an almost complete involution and disruption of the epithelium was shown by a fibrinoid type necrosis and the islands of epithelial cells lost in the tissue. Closer examination revealed tissue vacuolization, clefting, vesiculating

and ballooning cellular degeneration and intra-epithelial edema to the point of destroying the epithelial cells. In addition the underlying and supporting connective tissue was densely infiltrated with polymorphonuclear leucocytes and plasma cells. Vascular changes included vessel engorgement and extravascular clumping of erythrocytes.

Animal (#4) perfused with a solution of 1% collagenase also presented marked histologic inflammatory changes with more involvement on the experimental side (Figures 26, 27, experimental; 28, control). The main difference between this animal and the animal perfused with a 2% solution was the extent of the inflammatory response. The histologic changes were manifest in the same manner, only to a lesser degree. In both animals, collagenase appeared to be localized to the sulcular area as the cellular response was greatest in these locations.

In summary, it is interesting to note that no inflammatory response in the mesial sulcular gingivae accompanied the perfusion of water, buffer or hyaluronidase solutions. There were also no inflammatory changes in rats perfused with collagenase solutions for periods up to 60 hours. On the other hand, there were marked inflammatory changes in the intraoral tissues of rats perfused with collagenase solutions for a period of 96 hours.

CHAPTER V

DISCUSSION

The gingival tissues of all the rats, as observed by other investigators, exhibited evidence of mild inflammation. The typical low grade chronic inflammation might represent the activity of indigenous microorganisms of the rat gingiva or the action of proteolytic enzymes normally present in the oral fluids of the rat.

It is appropriate to discuss the methods of enzyme application reported in previous investigations. These methods may be grouped into three main categories: Needle injection, using intermittent application; needle injection with continuous flow of solutions; topical application of appropriate solutions.

Aisenberg (55) injected hyaluronidase into the interdental papillae of monkeys. Various time intervals were used. However, there was no mention of enzyme origin, concentration, injection rate, or enzyme deactivation factors.

Schultz-Haudt (56) injected hyaluronidase into the human gingival crevice by means of a "small syringe using extreme care to avoid injury to the tissue". No information with regard to enzyme concentration, origin, injection rate, or duration was cited.

Klingsberg (106) using rats, injected enzyme preparations three times per week for six weeks into the palatal interproximal papillae. Both hyaluronidase and collagenase were used, however these enzymes were not combined in any one animal. Enzyme concentration was 5% at a pH of

7.4. It is interesting to note an attempt to reduce tissue trauma by the reduction of injection intervals. Histologic evaluation revealed osteoclastic and collagenolytic activity combined with an increase in vascular exudate. There was no mention of enzyme preparation, or refrigeration of solutions. It is therefore difficult to assess the actual activity range of the preparations used.

Thonard (107) using mice, injected collagenase into the tissues. He noted that animals given a prolonged challenge of the enzyme showed an altered tissue response resembling chronic periodontitis. Again there was no mention of concentration, amount, source of enzyme used, or duration of the experiment.

Thilander (3) applied hyaluronidase to the facial and crestal aspect of the human gingival sulcus. The tissues had previously been dried with a combination "air blast" and filter paper strips placed into the sulcus. Application was repeated every five minutes for one hour. Histologic evaluation was limited to the crestal area of the gingival sulcus. Results indicated a widening of the intercellular spaces. However they suggested that this may be due to an increased uptake of water associated with the adhesive effect between epithelial cells. Alternatively, this might be due to the trauma caused by the initial air blast and filter paper insertion.

Stallard (78) endeavored to apply hyaluronidase topically to squirrel monkeys. Four quadrants were isolated with cotton rolls to dry the area prior to enzyme application. Hyaluronidase, 1200 U.S.P. units/cc was applied "topically" to the margin of the gingiva for a period of one hour per day for a total of two days. Trypan blue, a

vital dye, was likewise applied to the gingival margin for three hours. Results indicated only slight staining of the crevicular epithelium. Dye penetration was also noted within the intercellular cementing substance and deeper into the epithelial layers. However, Trypan blue staining for the most part was limited to the crevicular epithelium coronal to the epithelial attachment. The sulcular epithelium of the monkey does not exhibit a keratinized protective covering as does the rat. The possibility of trauma cannot be ruled out with the cotton roll application in the monkey as this may have caused excess loss of sulcular constituents making it possible for the infusion of specific agents to re-enter the depleated sulcular area. The enzymes used were not in a pure form, and may therefore have been contaminated with other proteolytic enzymes.

De Vincenzo (108) injected hyaluronidase and collagenase together into the interdental papillary area of the first and second molars in rats. This is the only controlled experimental situation to date utilizing a continuous flow system. The results indicate an extensive inflammatory response. However, the initial trauma of needle placement is a factor that must be evaluated.

Enzyme studies relating the tissue response to hyaluronidase and collagenase application to date indicate tissue trauma in one form or another resulting in a wide range of histopathologic changes. Assessment of the results of these studies is complicated by the likelihood of proteolytic (trypsin-like) enzyme contamination of the commercially available preparations and possible traumatic factors involved in delivery of the enzymes. It would seem useful to develop a system that

could deliver controlled quantities of solutions to localized intraoral areas in a variety of experimental animals.

Preliminary studies have indicated the experimental system used in this investigation to be atraumatic. For this reason, further study was directed toward the mesial sulcular area of the rats maxillary first molars.

Perfusion of the gingival tissues with hyaluronidase alone or with hyaluronidase and collagenase in sequence for periods up to 60 hours failed to elicit an inflammatory response. It is unlikely this failure can be attributed to denaturation of the enzymes within the apparatus. Temperatures of 4-7°C. were maintained inside the syringes during perfusion and the time of delivery of the solution through the tubing was only 18-27 minutes depending on the rate of perfusion.

The rat has an innate resistance to periodontal disease and also has an intact keratinized crevicular epithelium which may prevent penetrability of the area (109). Keratin is an albuminoid protein which is relatively insoluble and impermeable. It forms a protective sheet which preserves the fluid environment of the deeper layers of cells. Perhaps topical perfusion of the gingival epithelium might be more successful if made directly into the gingival sulcus in an experimental animal with unkeratinized epithelium such as the monkey.

It was significant that animals perfused with collagenase for four days revealed significant inflammatory gingival changes while animals perfused for two and one-half days or less showed no response. It again suggests the extended time required for adequate penetration of the epithelium. Like the observations of Thonard it might also mean that certain

host mechanisms are mobilized as part of the inflammatory response. When marked inflammation did occur it involved the control side as well as the experimental site. The intensity of inflammation, however, clearly diminished with the distance from the point of maximum enzyme concentration at the perfusion site. This observation and the fact that ten of the animals showed no evidence of an elicited inflammation strongly suggest that such factors as systemic host resistance, nutritional deprivation, oral hygienic status, surgical intervention, anesthesia, and needle trauma did not significantly affect the experimental situation. Indeed the animals appeared to adapt reasonably well to the experimental environment, and the apparatus effectively operated to permit the continuous and local atraumatic perfusion of test solutions.

Nevertheless further exploration of the experimental system is indicated for the study of various parameters. Another experimental animal such as the monkey could be chosen not only for its more convenient size but its nonkeratinized crevicular epithelium. The solutions should be delivered directly to the gingival sulcus with minimum occasion for dilution or extraneous distribution. The duration of perfusion should be adjusted to insure observation of any long-term effect. The enzyme solutions should be refrigerated in the range of 4-7°C. to minimize deactivation. Insulated teflon tubing might be used to aid in temperature control from delivery to perfusion site. Concentration of the enzyme preparation could be increased and flow rate decreased to maximize localization. Fresh enzyme solutions should be prepared every 4-6 hours in order to achieve maximum activity.

With appropriate adjustments, this experimental situation may well have significant potential in elucidating the role of collagenase and hyaluronidase and other local factors in the pathogenesis of periodontal disease. Native collagen is readily denatured at an acid pH and denatured collagen is susceptible to destruction by several proteases (6, 110). Thus the nonspecific proteolytic enzymes elaborated by such organisms as fusobacteria, spirochetes, veillonella and filaments in the oral cavity should also be tested for their role in the pathogenesis of periodontal disease (57, 61, 111-116).

CHAPTER VI

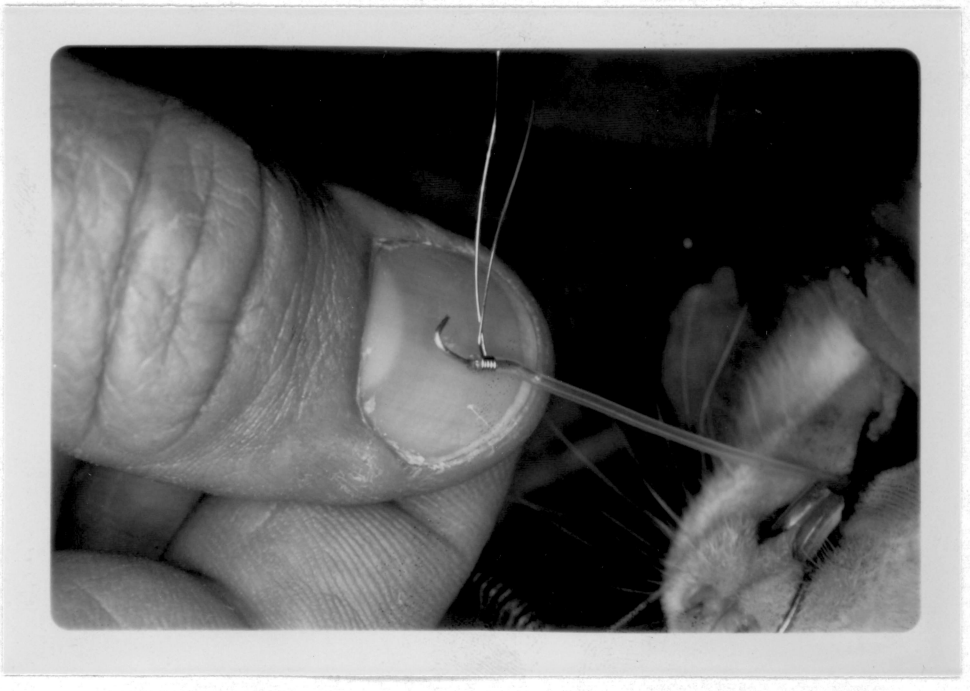
SUMMARY AND CONCLUSIONS

An experimental model was designed to elucidate the role of selected microbial enzymes in the pathogenesis of periodontal disease. The system consisted of an in vivo perfusion apparatus capable of continuously delivering controlled quantities of solutions to localized, contralateral, intra-oral areas in albino rats. The apparatus itself consisted of a perfusion pump connected by means of bilateral implanted tubing to needles fixed in close approximation to selected gingival surfaces. Histological examination has consistently shown the intra-oral needle placement to be atraumatic to the localized area of perfusion. Localization of the experimental solution was demonstrated in the gross specimen and histologically with Trypan blue.

Solutions of collagenase and hyaluronidase were perfused upon the mesial sulcular epithelium of the maxillary first molar while the appropriate control solutions were perfused on the opposite side. The continuous flow of hyaluronidase solution or a sequence of hyaluronidase and collagenase solutions each for 60 hours or less elicited little if any gingival inflammatory response in the albino rat. Histological examination revealed that perfusion with either 1% or 2% collagenase for a period of 96 hours induced the marked inflammatory changes.

Data obtained in this investigation suggest that the described apparatus should be useful in further studies of gingival disease. Each animal can act as its own control and the local factors associated with

dental plaque can be continuously and atraumatically delivered, individually or in combination at a controlled delivery rate to evaluate their effects on the gingival tissues. Future investigation of the relationship of the inflammatory potential of bacterial components associated with bacterial plaque may well be undertaken utilizing modifications of this apparatus.



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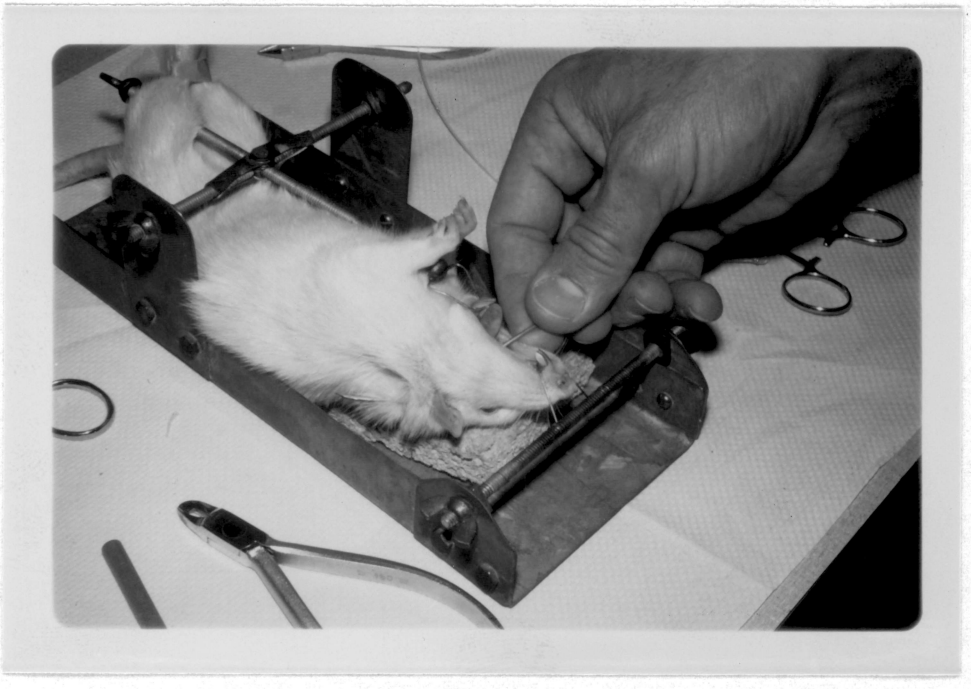

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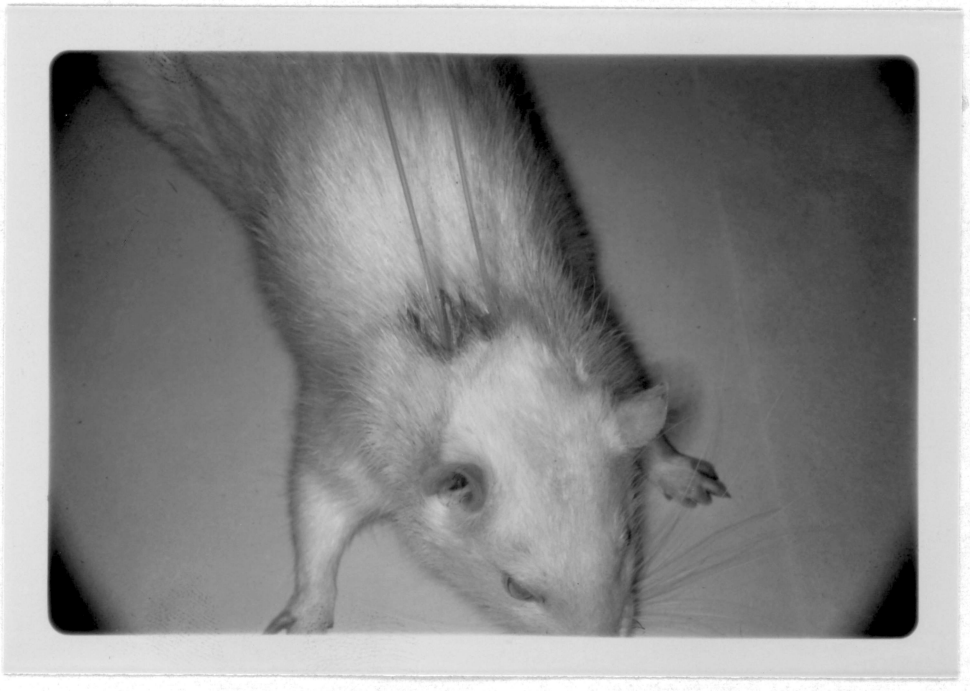

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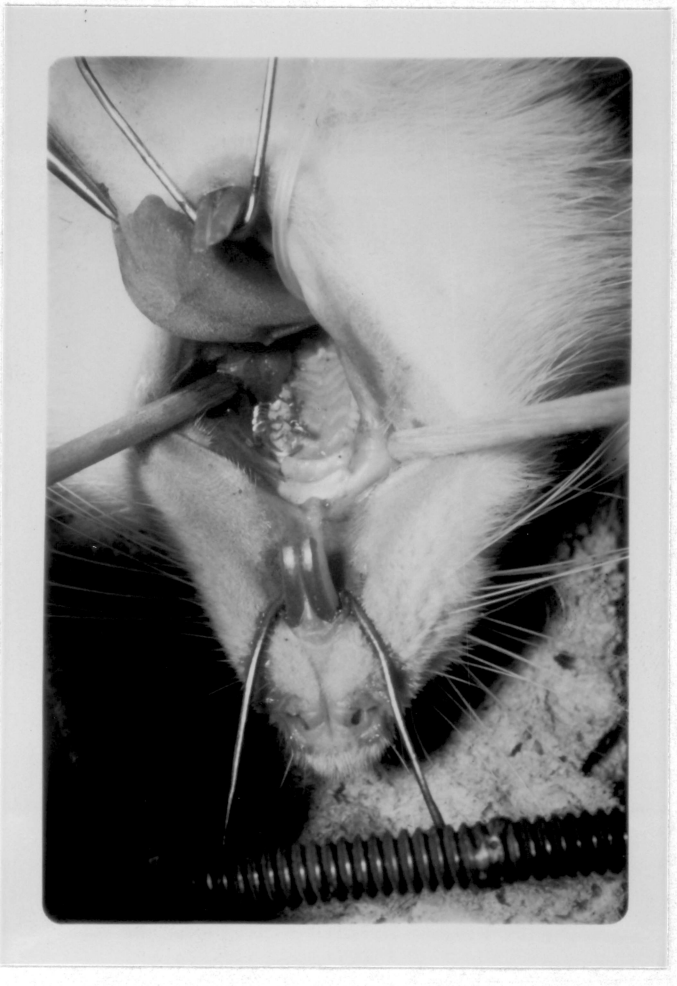

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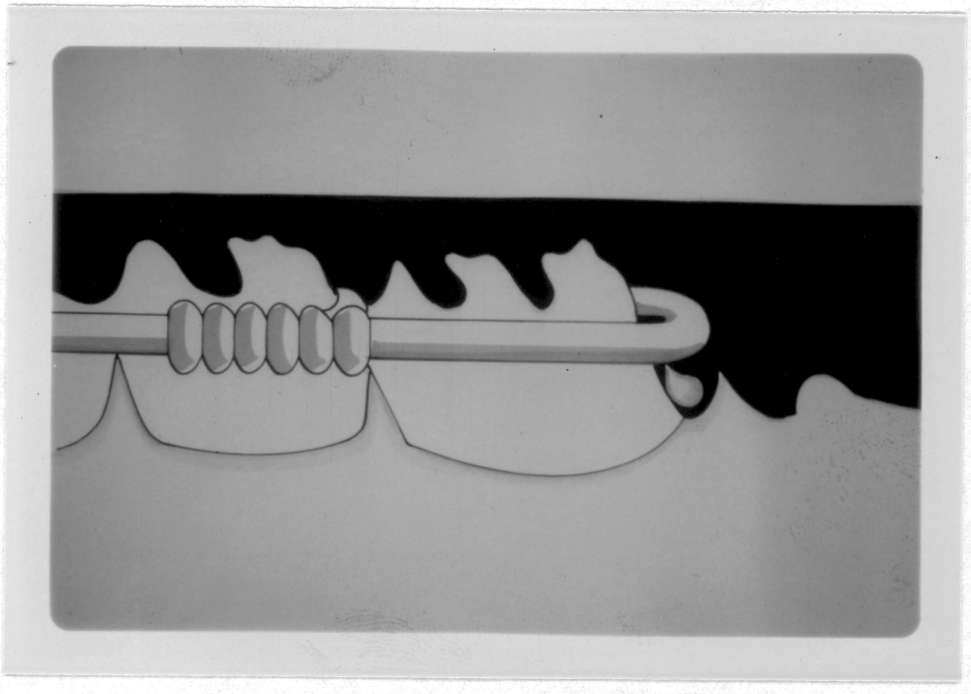

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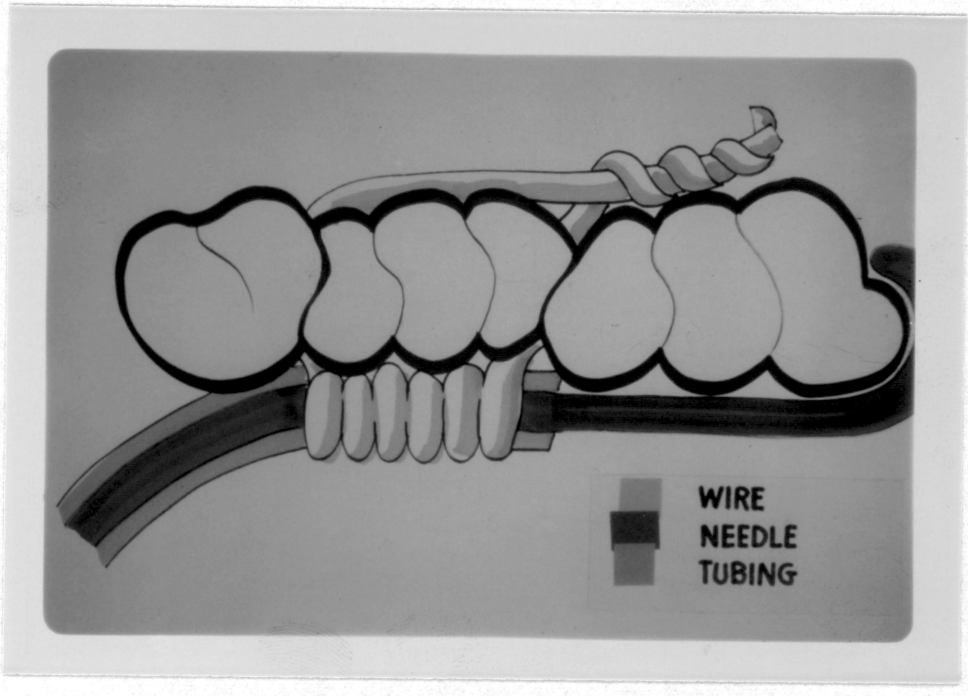

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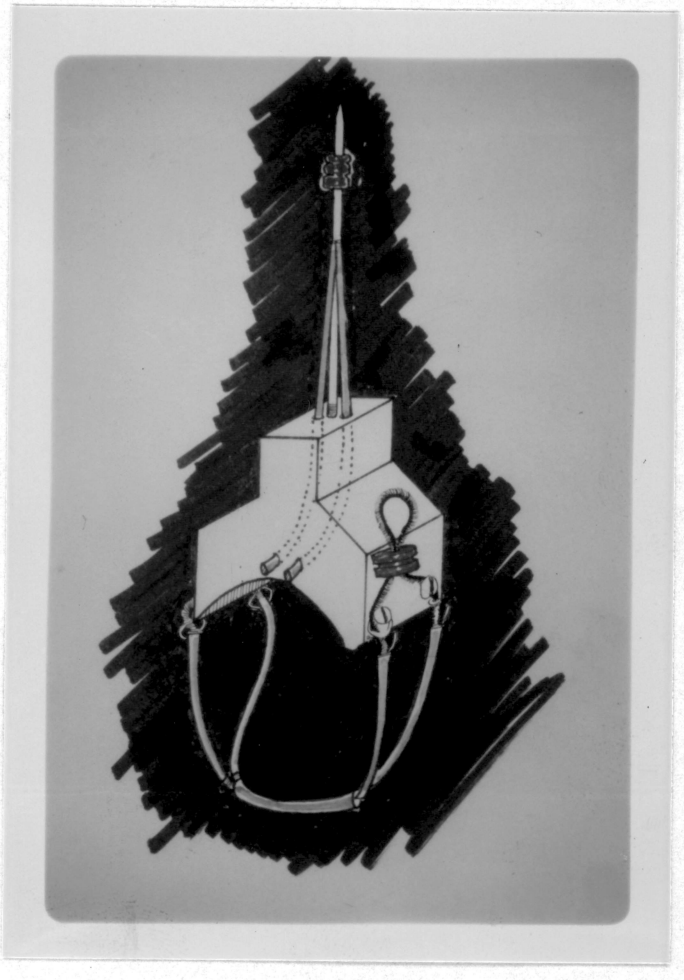

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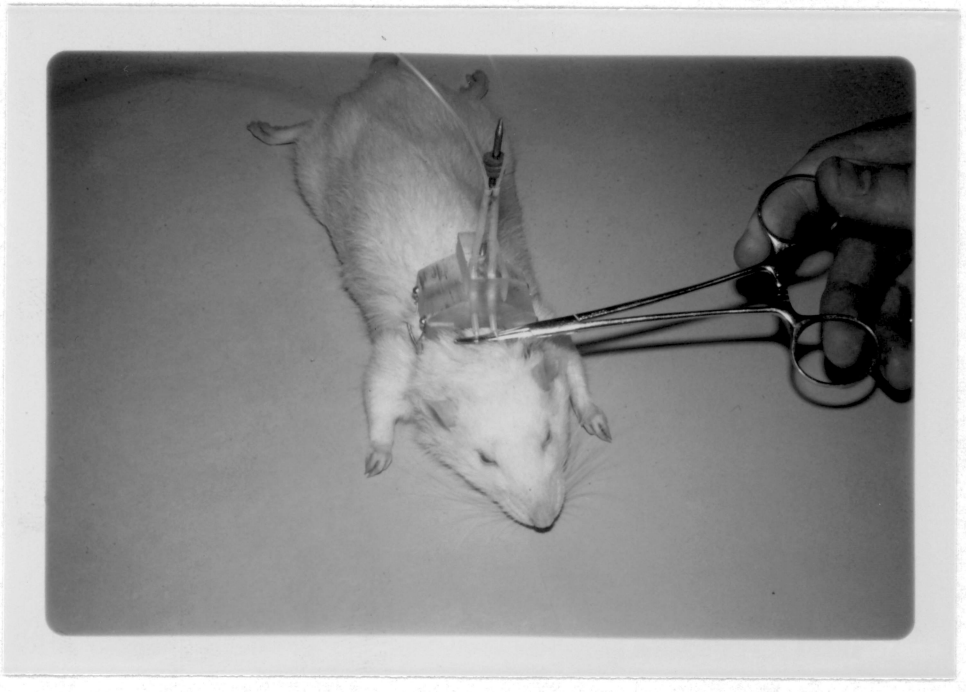

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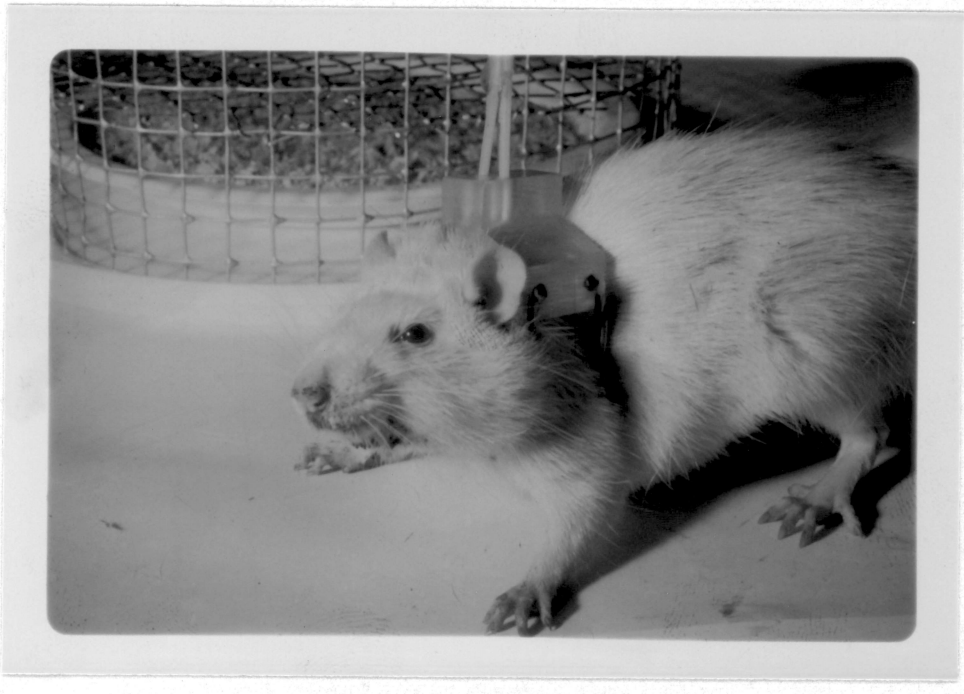

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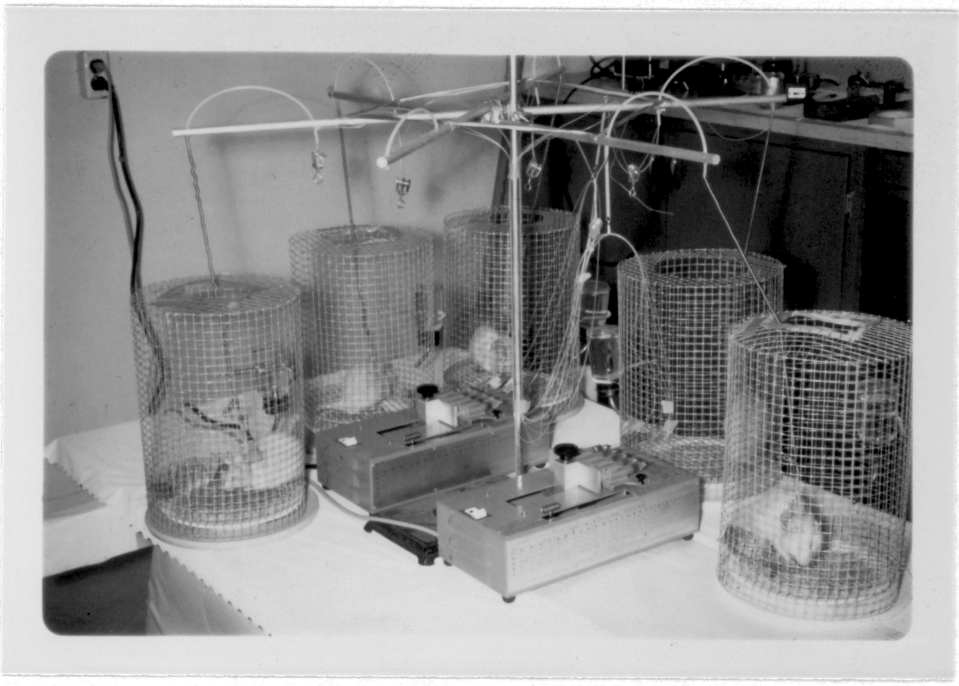

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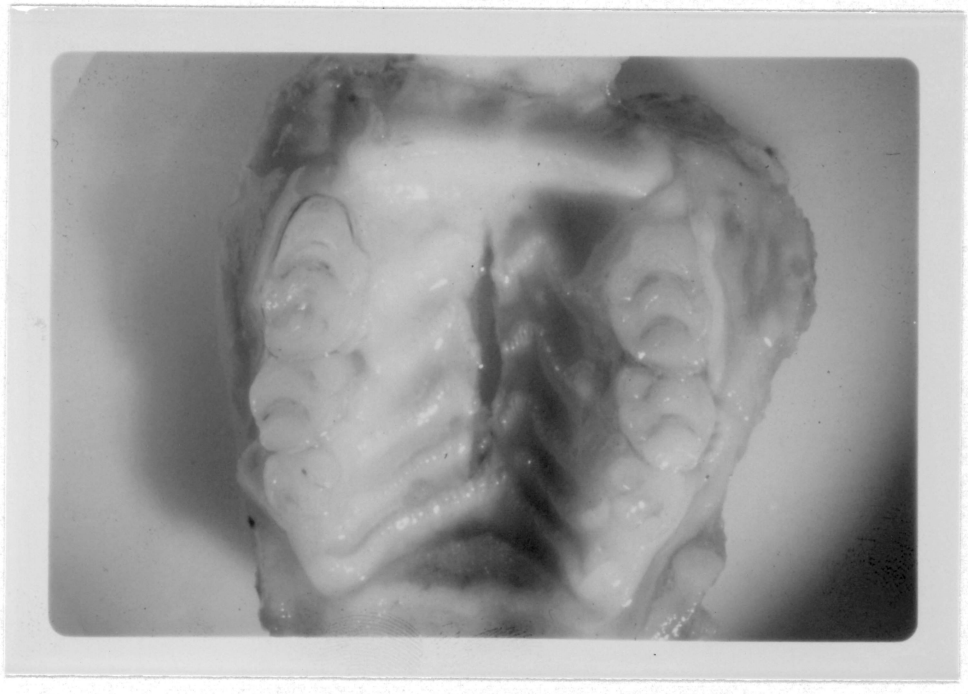

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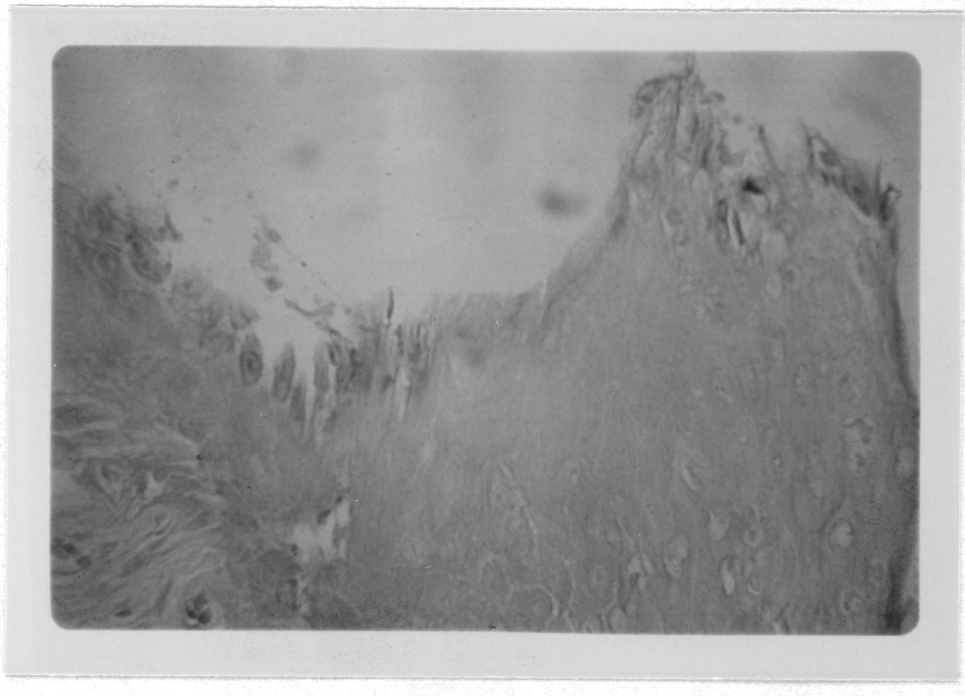

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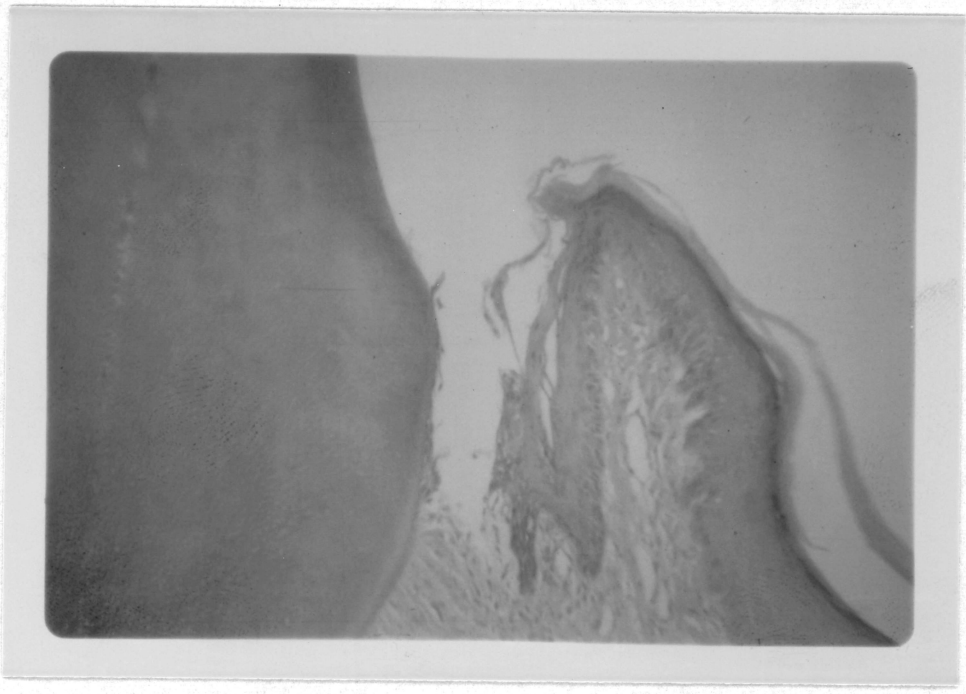

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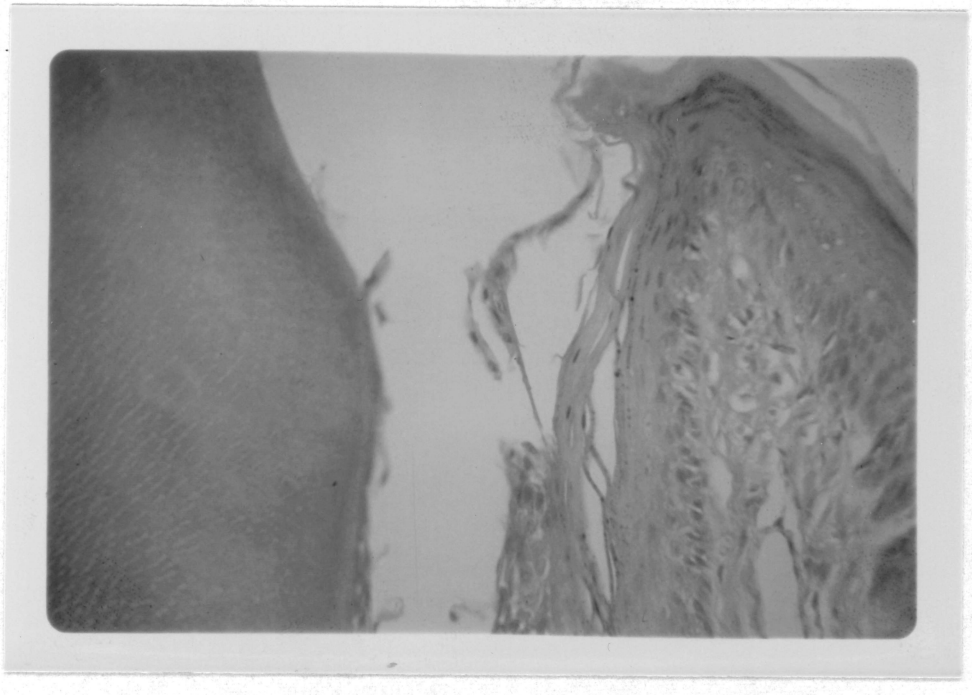

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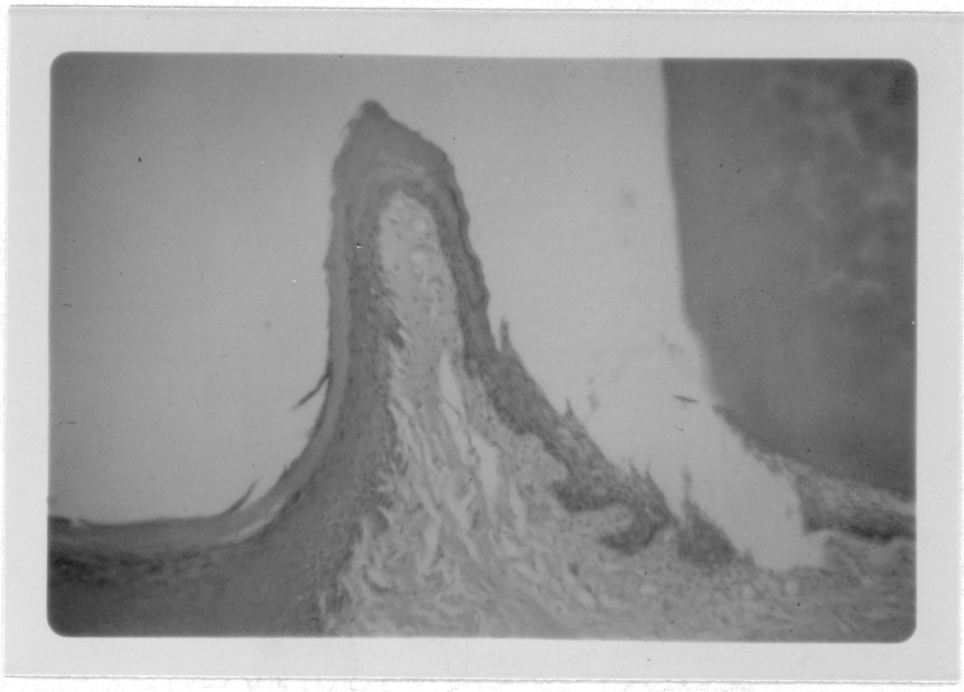

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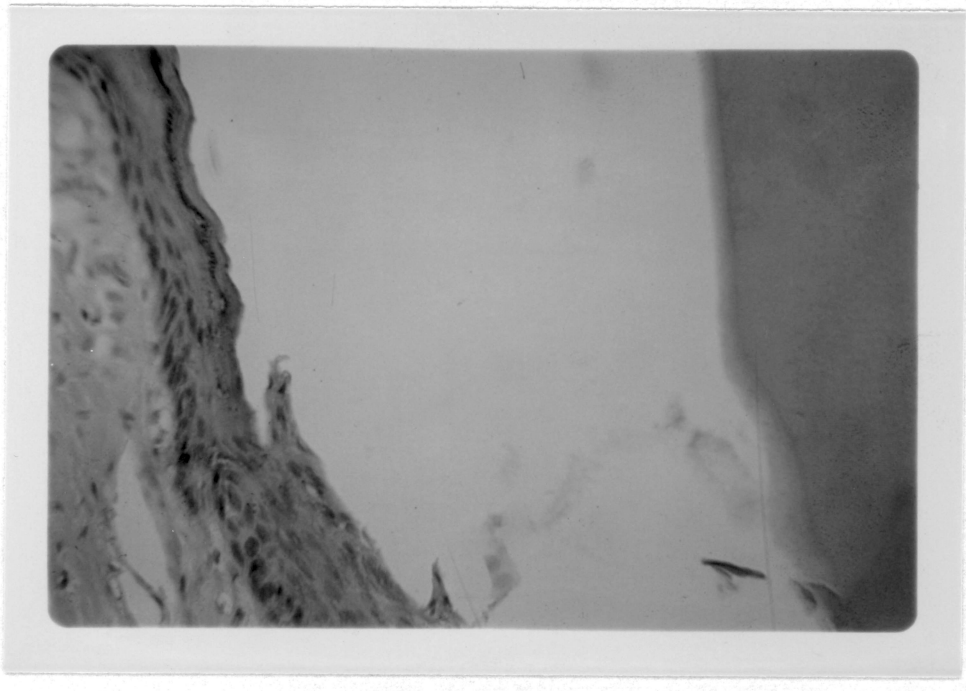

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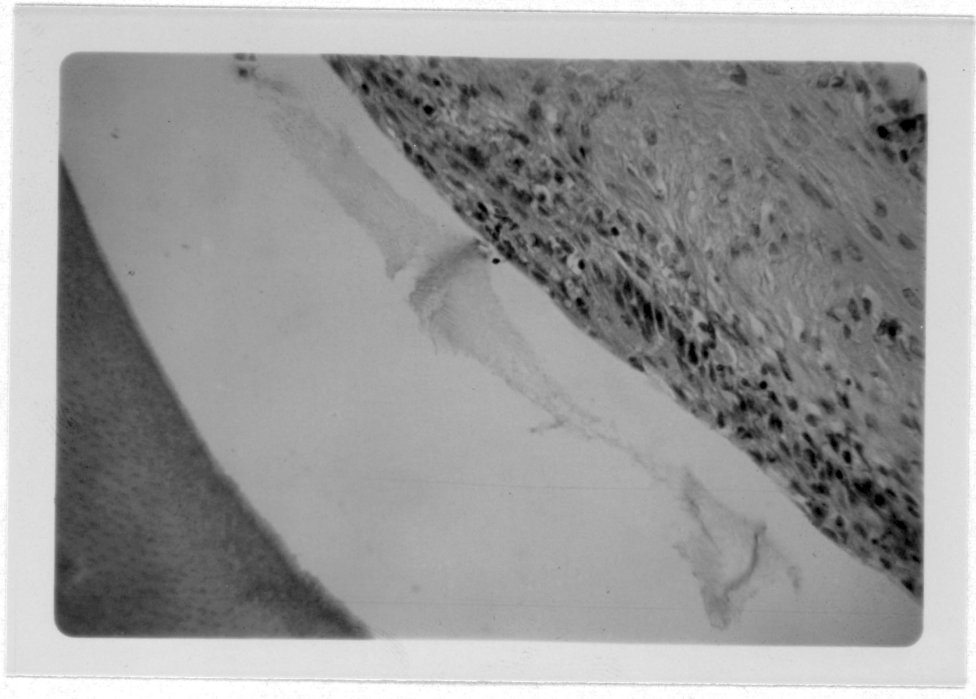

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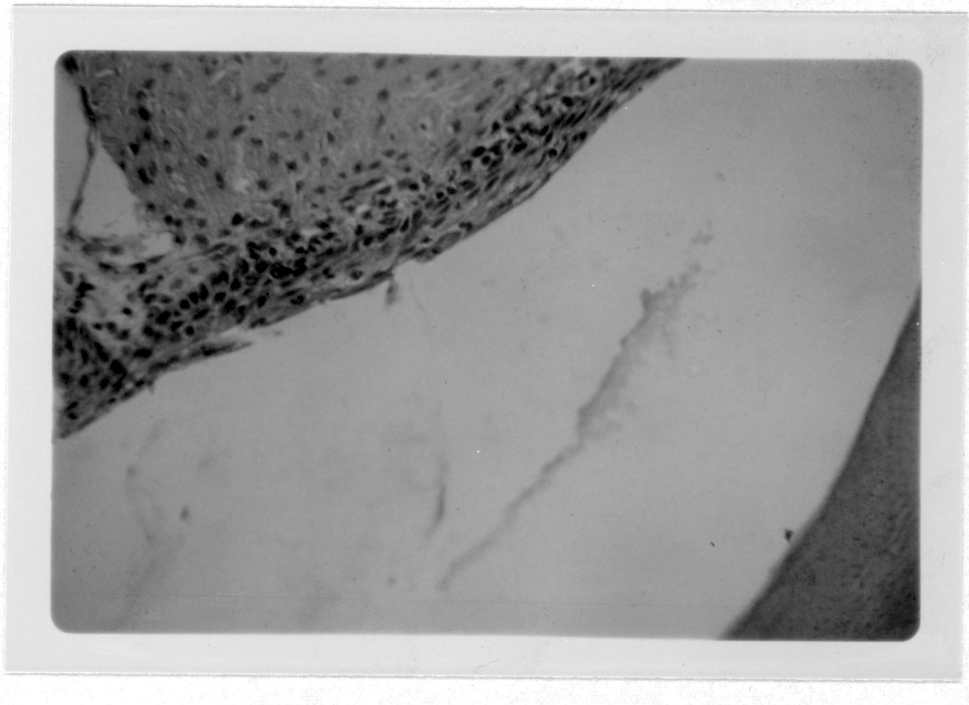

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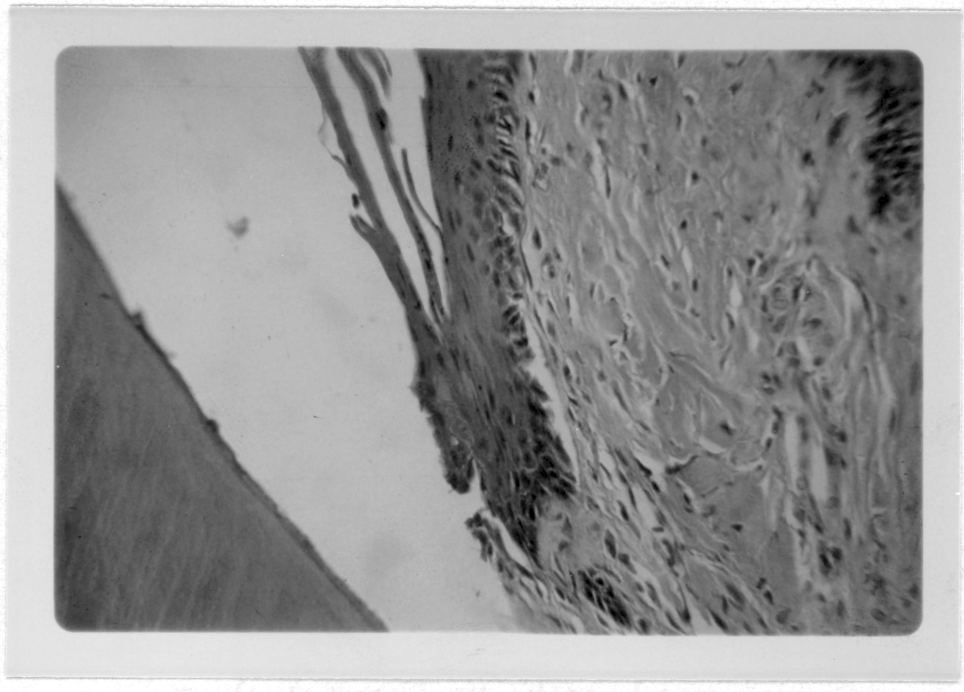

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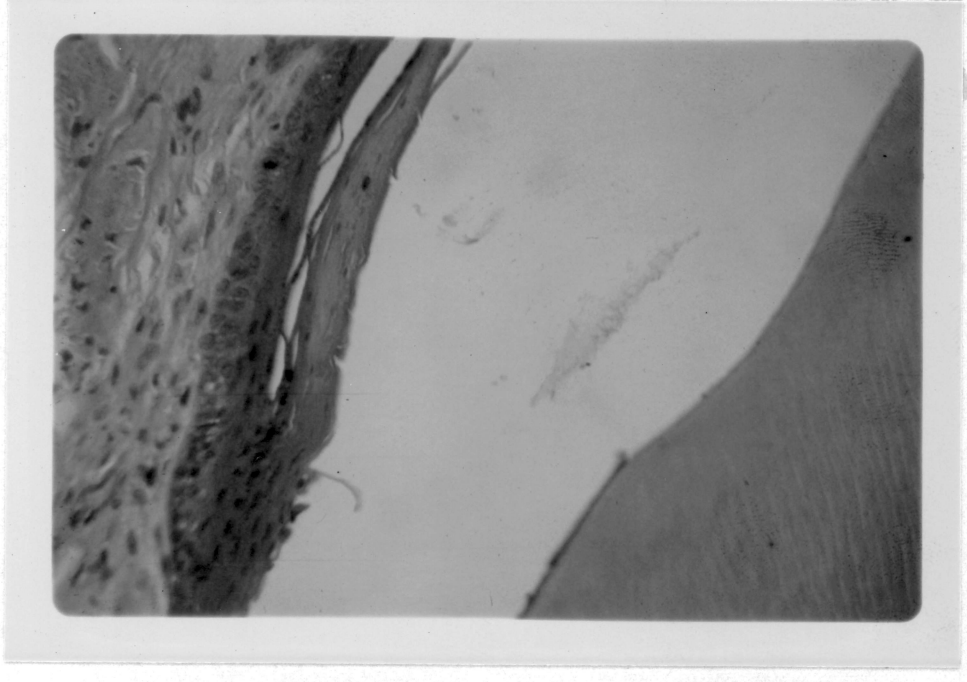

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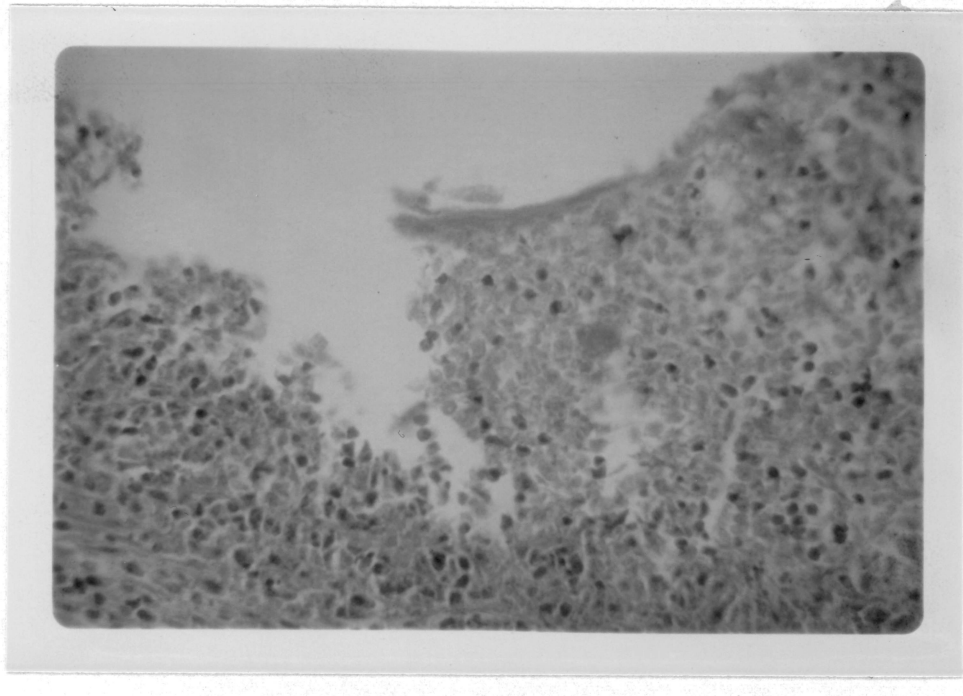

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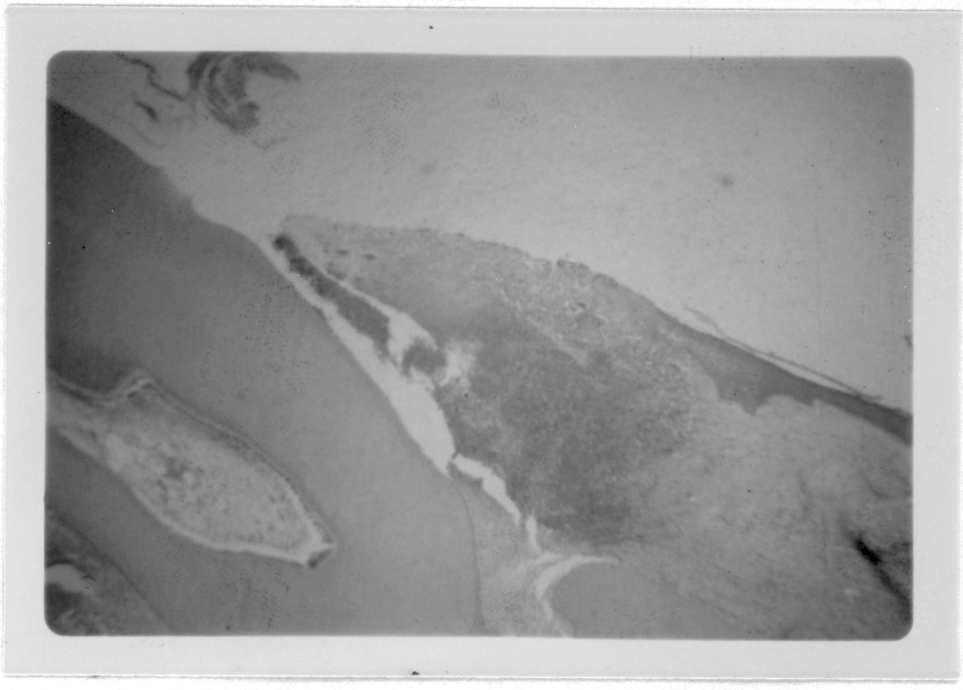

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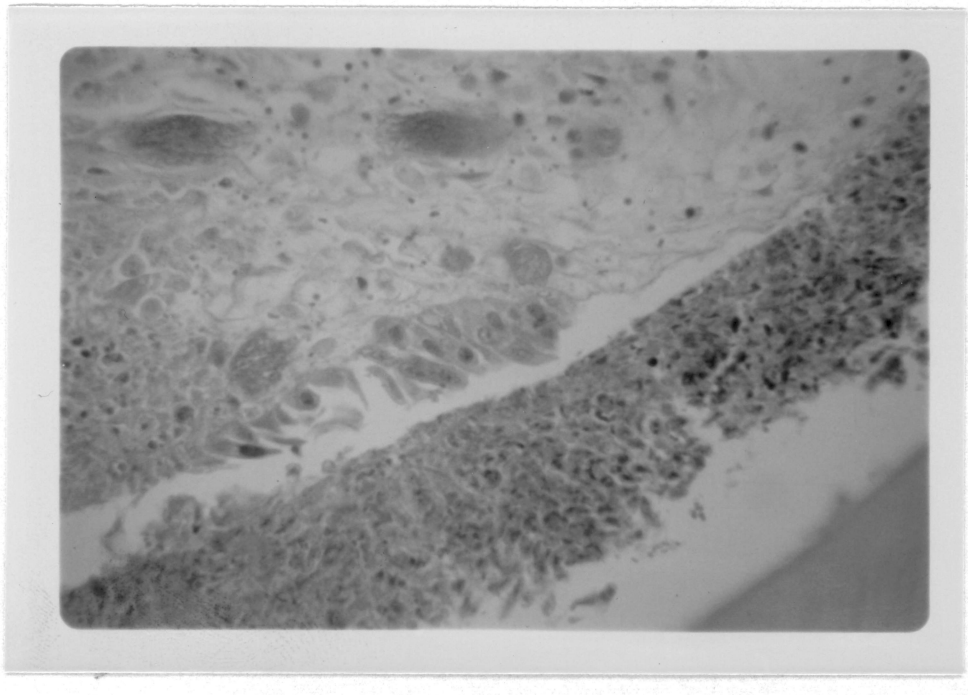

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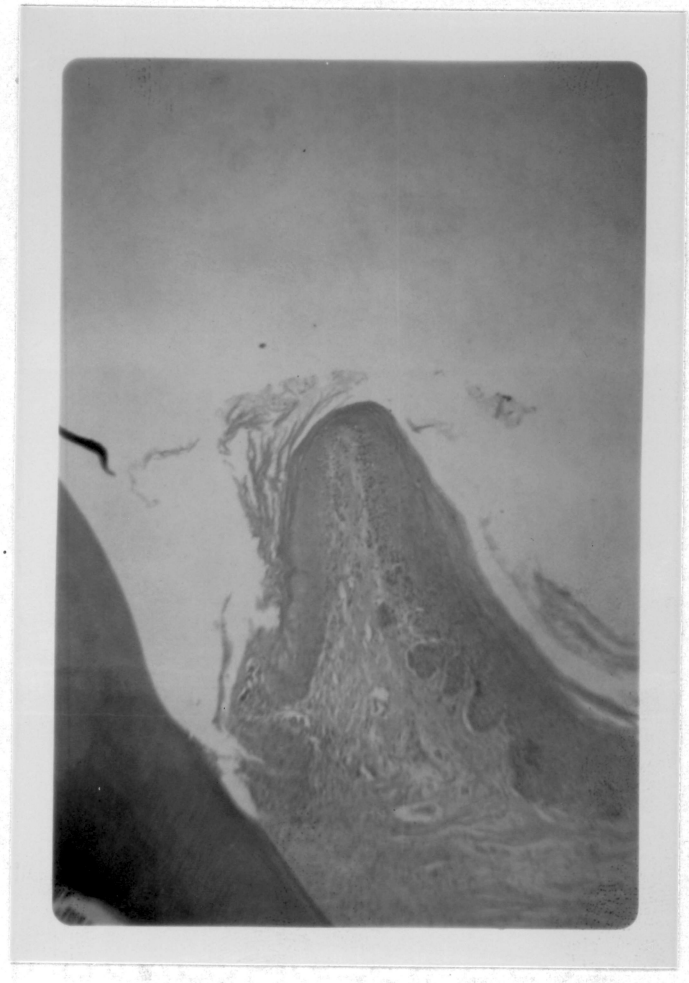

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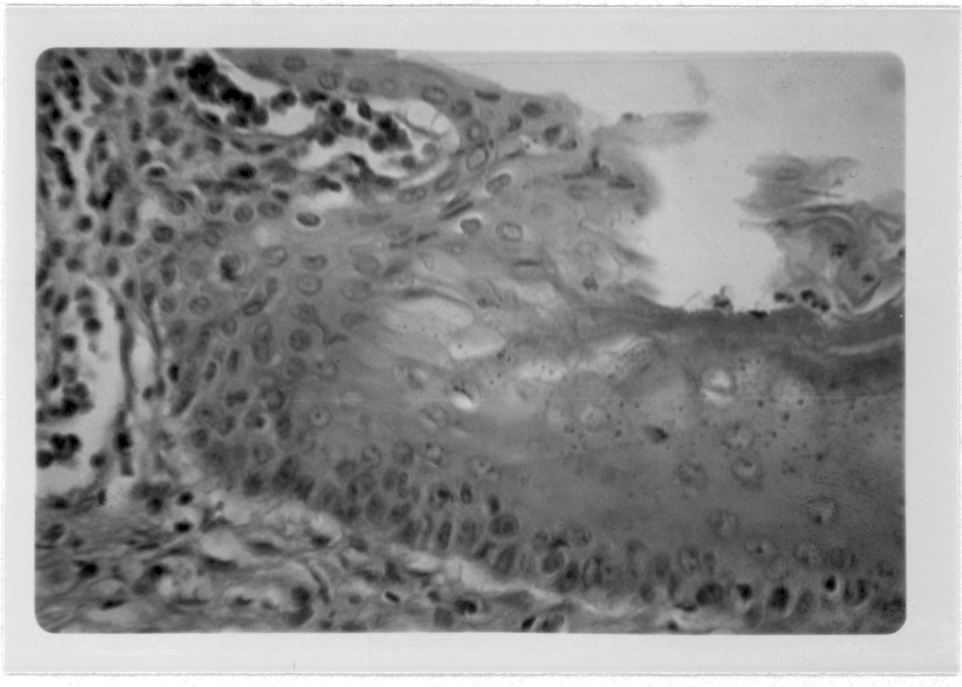

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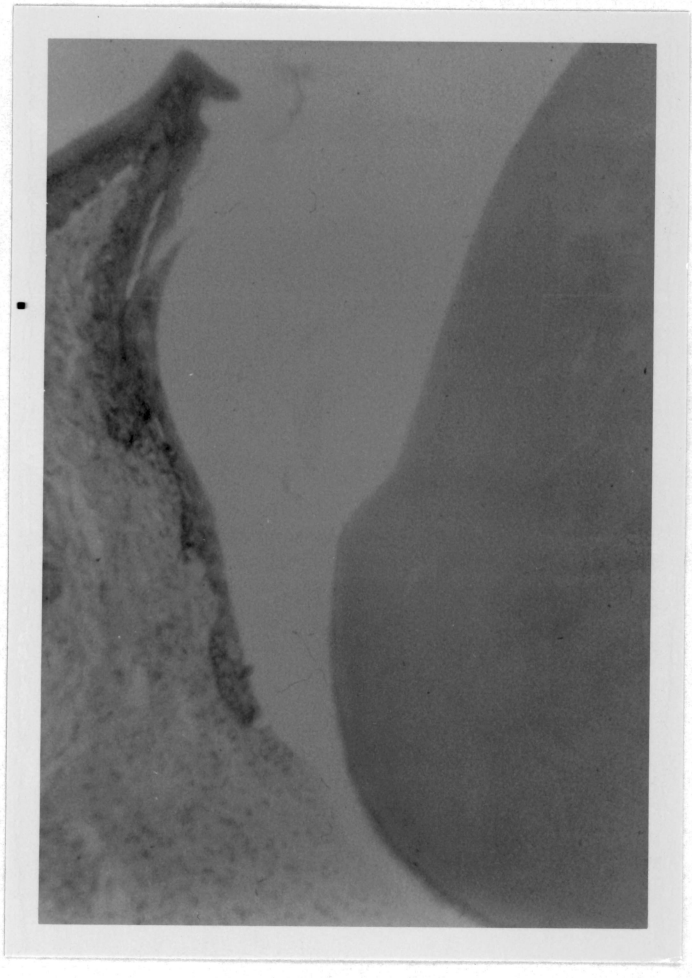

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TABLE I

A SUMMARY OF THE EXPERIMENTAL PROTOCOL

Animal Number	Side	Solution	Rate ml/day	Perfusion Solution	Time (hrs.) Dye	Inflammation
1	Exp.	Tap water	4.8	19	5	+
	Cont.	None			5	
2	Exp.	Tap water	4.8	91	5	+
	Cont.	None			5	
3	Exp.	Tap water	4.8	163	5	+
	Cont.	None			5	
4	Exp.	1% Collagenase	4.8	96	2	++
	Cont.	Tris Buffer	4.8	96	2	
5	Exp.	1% Hyaluronidase	4.8	96	2	+
	Cont.	Deionized water	4.8	96	2	
6	Exp.	0.5% Collagenase	4.8	48		
		0.5% Hyaluronidase	4.8	48	2	
	Cont.	Tris Buffer	4.8	48		+
		Deionized water	4.8	48	2	
7	Exp.	1% Hyaluronidase	5.4	36		
		1% Collagenase	5.4	36	2	
	Cont.	Deionized water	5.4	36		+
		Tris Buffer	5.4	36	2	
8	Exp.	1% Hyaluronidase	5.4	60		
		1% Collagenase	5.4	60	2	
	Cont.	Deionized water	5.4	60		+
		Tris Buffer	5.4	60	2	
9	Exp.	1% Hyaluronidase	5.4	36		
		1% Collagenase	5.4	36	2	
	Cont.	Deionized water	5.4	36		+
		Tris Buffer	5.4	36	2	
10	Exp.	2% Collagenase	7.1	96	2	+++
	Cont.	Tris Buffer	7.1	96	2	
11	Exp.	2% Hyaluronidase	7.1	96	2	+
	Cont.	Deionized water	7.1	96	2	
12	Exp.	2% Hyaluronidase	7.1	48		
		2% Collagenase	7.1	48	2	
	Cont.	Deionized water	7.1	48		+
		Tris Buffer	7.1	48	2	

Degree of Inflammation: Mild +; Moderate ++; Severe +++.

LOMA LINDA UNIVERSITY

Graduate School

AN IN VIVO PERFUSION APPARATUS FOR STUDIES ON GINGIVAL DISEASE IN THE ALBINO RAT: ASSESSMENT BY A PERFUSION OF THE ENZYMES COLLAGENASE AND HYALURONIDASE

By

James A. Smith

An Abstract of a Thesis in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Field of Periodontics

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June 1970

A B S T R A C T

ABSTRACT

This thesis describes an in vivo perfusion apparatus which can continuously deliver controlled quantities of solutions to localized, contralateral intraoral areas in albino rats. Three animals were perfused on the experimental area (the mesial sulcular area of the maxillary first molar) on one side with tap water and Trypan blue. Localization of the solution delivered and minimal histological changes due to the presence of the apparatus was demonstrated on these animals. Solutions of .5%, 1%, and 2% hyaluronidase and collagenase were perfused on the experimental areas of nine animals. When both enzymes were used, hyaluronidase always preceeded collagenase in sequence. Rate of flow ranged from 4.8 to 7.1 ml/day. The appropriate control solutions were delivered on the contralateral side of each animal undergoing enzyme perfusion. Data obtained from animals perfused with collagenase for periods over sixty hours at from 1% to 2% concentration indicated moderate to severe inflammatory changes on the experimental side. More meaningful results should be obtained with the modifications discussed in this report.