An In Vivo Perfusion Apparatus for Studies on Gingival Disease: Assessment by a Perfusion of Nonspecific Proteolytic Enzymes

Don Dornan

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AN IN VIVO PERFUSION APPARATUS FOR STUDIES ON GINGIVAL DISEASE:
ASSESSMENT BY A PERFUSION OF NON SPECIFIC PROTEOLYTIC ENZYMES

By
Don Dornan

A Thesis in Partial Fulfillment of the
Requirements for the Degree of Master of Science
in the Field of Periodontics

May 1969
155249
Each person whose signature appears below certifies that he has read this thesis and that in his opinion it is adequate, in scope and quality, as a thesis for the degree of Master of Science.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION AND STATEMENT OF PROBLEM</td>
<td>1</td>
</tr>
<tr>
<td>II. REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>Bacterial Invasion</td>
<td>5</td>
</tr>
<tr>
<td>Enzymes</td>
<td>6</td>
</tr>
<tr>
<td>Histamine and Bradykinin</td>
<td>12</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>14</td>
</tr>
<tr>
<td>Antigen-Antibody Hypersensitivity</td>
<td>15</td>
</tr>
<tr>
<td>Endotoxins</td>
<td>16</td>
</tr>
<tr>
<td>III. METHODS AND MATERIALS</td>
<td>18</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>24</td>
</tr>
<tr>
<td>Effects of Apparatus on the Experimental Animal</td>
<td>24</td>
</tr>
<tr>
<td>Localization of the Solution Delivered</td>
<td>24</td>
</tr>
<tr>
<td>A. Gross Observations</td>
<td>24</td>
</tr>
<tr>
<td>B. Microscopic Observations</td>
<td>25</td>
</tr>
<tr>
<td>Histological Observations</td>
<td>25</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>28</td>
</tr>
<tr>
<td>Enzyme Selection</td>
<td>28</td>
</tr>
<tr>
<td>Perfusion Parameters</td>
<td>30</td>
</tr>
<tr>
<td>Animal Response</td>
<td>31</td>
</tr>
<tr>
<td>Histological Evaluation</td>
<td>31</td>
</tr>
<tr>
<td>Comparison With Other Enzyme Investigations</td>
<td>33</td>
</tr>
<tr>
<td>Suggestions for Future Studies</td>
<td>35</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>PAGE</td>
</tr>
<tr>
<td>-----------------</td>
<td>------</td>
</tr>
<tr>
<td>VI. SUMMARY AND CONCLUSION</td>
<td>37</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>40</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>47</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HISTOLOGIC APPEARANCE OF THE MESIAL SULCULAR AREA.</td>
<td>47</td>
</tr>
<tr>
<td>2. A SUMMARY OF THE EXPERIMENTAL PROTOCOL AND THE WEIGHT CHANGES.</td>
<td>48</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>49</td>
</tr>
<tr>
<td>2.</td>
<td>49</td>
</tr>
<tr>
<td>3.</td>
<td>50</td>
</tr>
<tr>
<td>4.</td>
<td>51</td>
</tr>
<tr>
<td>5.</td>
<td>52</td>
</tr>
<tr>
<td>6.</td>
<td>52</td>
</tr>
<tr>
<td>7.</td>
<td>53</td>
</tr>
<tr>
<td>8.</td>
<td>54</td>
</tr>
<tr>
<td>9.</td>
<td>55</td>
</tr>
<tr>
<td>10.</td>
<td>56</td>
</tr>
<tr>
<td>11.</td>
<td>57</td>
</tr>
<tr>
<td>12.</td>
<td>58</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION AND STATEMENT OF PROBLEM

The relationship of gingival deposits to inflammatory periodontal disease is one of unlimited dimensions. Biochemical and microbial research has indicated that the toxic bacterial products associated with dental plaque provide the potential to elicit or modify an inflammatory response. Waerhaug states, "When the bacterial plaque has penetrated below the gingival margin, the soft tissue will lie in direct contact with the irritating bacteria, and they and their products cause inflammation in the connective tissue and migration of leucocytes through the epithelium." Chronic inflammatory responses of the periodontium may result in breakdown of the periodontal fibers, apical migration of the epithelial attachment and alveolar bone resorption.

Two questions concerning the relationship of bacteria to periodontal disease are of major interest. The first deals with the parameters that make an organism capable of producing periodontal disease such as ability to form dental plaque. The second question is: How do the bacteria or their products destroy the periodontal structures? The initiation of inflammation by bacterial products has been studied by a number of investigators. They have taken various enzymes and toxins associated with plaque and either injected or intermittently applied them to gingival tissues in experimental animals. With the injection technique it is difficult to differentiate the effects of physical trauma from that of the
solution injected. Since human dental plaque is often continuously rather than intermittently present it would be of interest to have an application technique which could better simulate the natural phenomenon.

This thesis will describe an in vivo perfusion apparatus which can continuously deliver controlled quantities of solutions to localized, contralateral intra-oral areas in rats with minimal tissue damage. In order to produce a chemical change in the subepithelial connective tissue the experimental solutions must penetrate the crevicular epithelium. The most logical place for this penetration would be through the intercellular spaces. Since the intercellular substance between epithelial cells contains protein, non specific proteolytic enzymes were used in the perfusion. These proteolytic enzymes also have the capacity to activate the chemical mediators of inflammation. The means of assessing the performance of the apparatus was by gross and histological observations.
CHAPTER II

LITERATURE REVIEW

Current research has provided ample evidence that dental plaque is associated with inflammatory gingival disease. Patients with periodontal disease demonstrate a greater accumulation of dental plaque than those without the disease. Removal of this plaque results in diminution of gingival inflammation. Furthermore, patients with clinically healthy gingiva develop gingivitis by stopping normal hygiene and allowing the accumulation of plaque. When these patients resumed oral hygiene, their gingiva returned to clinical health. Plaque formation begins with the deposition of salivary glycoproteins onto the enamel surface forming a thin pellicle. After 24 hours the pellicle is progressively invaded by bacteria which are capable of producing extracellular polysaccharides. These polysaccharides form an intermicrobial matrix and may aid in adhesion of bacteria to teeth and protect them from being shed into saliva. Mature dental plaque is a sticky mass comprised mainly of bacteria which can serve as a matrix for mineralization between the inner plaque surface and the tooth to form dental calculus.

Many investigators have observed microorganisms in the gingival sulci of both healthy and diseased gingival tissue. The specific mechanism by which these microorganisms change the environment in the sulcus to initiate inflammation is not firmly established. In the healthy gingival sulcus the microorganisms have a commensalastic association with the host. It has been proposed that in periodontal disease the delicate
balance that exists between the host and the microorganisms is disturbed. This disturbance may occur either by an increase in the susceptibility of the host or by an increase in the aggressive nature of the bacteria in the sulcus. Virulence, invasiveness, and toxigenicity are bacterial properties which may account for the aggressive bacterial change.

The inflammatory response is described as a cellular reaction to injury and is generally characterized by a complex series of vascular changes, the exudation of fluid and cells, and repair. A bacterial insult to cells is capable of injuring these cells causing a release of intracellular chemicals which may mediate and govern the process of inflammation. Recently, with the use of more sophisticated techniques, investigators have been able to reach a better understanding of this complex inflammatory process at a subcellular level of organization. New questions are being asked concerning which specific bacteria or bacterial products of dental plaque have the potential to elicit an inflammatory response of the periodontium. If inflammation is not elicited by bacteria, per se, then the products associated with bacteria should receive special attention. It will be the purpose of this literature review to discuss possible mechanisms by which dental plaque may produce gingival inflammation. These potential mechanisms include: bacterial invasion, bacterial enzyme activity, histamine and bradykinin systems, lysosomal activity, antigen-antibody hypersensitivity, and endotoxin activity. The apparatus described in this thesis could be useful for further study of these mechanisms.
BACTERIAL INVASION

Bacteria may initiate gingival disease by invasion through the sulcular epithelium into the underlying connective tissue. In 1950, Box reported a fungus-like organism in the connective tissue of the gingiva. He believed these organisms were associated with breaks in the lining of the sulcular epithelium.

Haberman has studied gingivectomy specimens with special bacterial stains to demonstrate bacterial invasion. He stated that "microorganisms do invade the gingival tissues, using the lymphatics and perivascular route as a means of penetration." Haberman did not mention the number of sections in which bacteria were found, nor did he clearly elucidate the inflammatory state of the tissue during removal.

Courant and Brader utilized a tissue sectioning technique, starting through the cut gingival surface first to eliminate the possibility of bacteria being pulled from the sulcular epithelial tissue into the deeper tissues by the sectioning blade. They showed Bacteriodes melaninogenicus and collagenase in the connective tissue of gingival biopsies from inflamed human tissue. The possibility that bacteremic blood and saliva could contaminate the sections was not eliminated by their technique. These patients had been scaled one to three months prior to excision and no unscaled controls or uninflamed tissues were used.

The electron microscopic work by Listgarten and Socransky on tissue specimens from patients with acute necrotizing ulcerative gingivitis, has shown intermediate spirochetes in almost pure culture in the
connective tissue. Intermediate spirochetes were also found in the
dental plaque adjacent to the gingival sulcus. The spirochetes were
not found in the plaque of the healthy patients used as controls.
Whether these spirochetes were present as a result of contamination
during sectioning or by virtue of a more favorable environment from the
initial lesion or by invasion of the sulcular epithelium remains un-
determined.

Bibby\textsuperscript{17} stated that the toxic products from the bacteria in the
sulcus irritate the gingival tissues without bacterial invasion of the
deeper tissues. His proposal has gained more support by recent experi-
mental evidence.\textsuperscript{18,19,20} Wertheimer\textsuperscript{18} has shown that bacterial activity
is limited to the superficial layers of the crevicular epithelium and
that it is the occasional microbe that is found in the subepithelial
connective tissue. Freedman, et al.,\textsuperscript{19} studied the ultrastructure of
chronically inflamed human gingiva. Bacteria were not seen between the
epithelial cells nor in the connective tissue in spite of a widening of
epithelial intercellular spaces in the sulcular epithelium. The work of
Gibson and Shannon\textsuperscript{20} demonstrated bacterial presence in 60\% of inflamed
tissue from those patients with oral prophylaxis 24 hours prior to sur-
gery. Only 10\% of the inflamed tissue removed without the prior pro-
phylaxis exhibited bacteria in the connective tissue. It was their con-
tention that tissue trauma rather than actual bacterial invasion accounted
for bacteria in the tissues.

\textbf{ENZYMES}

Schultz-Haudt\textsuperscript{21,22,23} and others\textsuperscript{24,25} have contended that bacteria
present in the gingival crevice produce destructive toxins which are
etiological factors in the development of gingival inflammation. Hyaluronidase and proteolytic enzymes are produced by the microbial population in dental plaque. It has been proposed that these enzymes may be responsible for detachment and penetrability of the sulcular epithelium. Hence, the subepithelial connective tissue may be subject to insult via the penetration of crevicular epithelium by deleterious microbial products.

The possibility that the crevicular epithelium can act as a semi-permeable membrane by allowing, under certain conditions, the passage of fluids in either direction has been the subject of much research. With healthy tissue, free passage of water and electrolytes is normal, but only a small number of plasma proteins permeate the walls of capillaries and venules. It is well established that with inflamed tissue there is a positive flow of plasma and proteins from the connective tissue through the sulcular epithelium into the gingival sulcus.

The sulcular epithelium may provide only fragile resistance to the passage of fluids and particulate matter. Ratcliff has demonstrated, after intravenous injections in rats, the movement of relatively large carbon particles from blood vessels through the intercellular spaces of their intact sulcular epithelium. He speculates that intact sulcular epithelium may not act as a barrier to fluids and particulate matter moving in and out of the sub-epithelial connective tissue. Egelberg has shown that topically applied histamine will diffuse through the gingival epithelium into the subepithelial connective tissue. Fine, et al., have demonstrated that carbon particles of 1-3 microns, when gently applied to the crest of the free gingiva, can be found consistently in the
underlying connective tissue of clinically normal and periodontally involved humans. They also demonstrated the carbon particles in the macrophages of the connective tissue. This should eliminate contamination, by virtue of sectioning technique as a factor.

The large number of streptococci in plaque and debris which are capable of producing hyaluronidase and proteolytic enzymes is another possible etiologic factor mentioned by many. Rovelstadt, et al., investigated the metabolic differences in the bacterial debris from the gingival crevices of patients with periodontal disease and healthy patients. They found that hyaluronidase activity per mg. of debris was the same for both diseased and healthy tissue. This did not imply that the total amount of hyaluronidase was the same, however, since the afflicted individuals accumulated a great deal more debris.

Mucopolysaccharides are thought to contribute to the adhesion between epithelial cells and between these cells and hard surfaces. Hyaluronidase will depolymerize hyaluronic acid and Chondroitin sulfate A and C, some of the major constituents of ground substance, and thus give rise to increased tissue permeability. Thilander has somewhat substantiated this effect of hyaluronidase on epithelium in the gingival sulcus. Using the electron microscope, he demonstrated a widening of the intercellular spaces of human gingival epithelium after local application of this enzyme.

More evidence for the feasibility of exogenous penetration of the epithelium has been provided by Murphy and Stallard. They have demonstrated the penetration of trypan blue between epithelial cells after
topical application of hyaluronidase. These results led the authors to postulate that the dye diffusion was due to hyaluronidase depolymerizing the intercellular cementing substance (Chondroitin sulfate A and C) which ultimately decreased its viscosity and increased tissue permeability.

Loe has stated that there has been no real scientific study showing the chemical components of epithelial intercellular substance. It is known, however, that the intercellular substance contains proteins. Epithelial cells are thought to adhere to hard surfaces and to each other by physical-chemical forces from the glycoproteins of the cell surface. Proteolytic enzymes such as trypsin decrease this adherence. Bacterial proteases from Fusobacteria Filaments, Spirochetes, and Veillonella have been demonstrated in the oral cavity. Schultz-Haudt, et al., proposed that proteolytic enzymes produced by the microbial flora, may be responsible for epithelial penetrability by reducing the adhesion of epithelial cells to the tooth surface and to each other.

Klingsberg and Butcher were interested in the effects that various enzymes would have on the connective tissue. They found that hyaluronidase and collagenase were relatively inaffective agents in promoting connective tissue lysis. A pronounced connective tissue lysis was noted with proteolytic enzymes such as papain and trypsin. Since all enzymes were injected repeatedly, interpretation of the meaning of their results is difficult due to the tissue trauma inherent in their method of application.

Collagenase, a product of the Bacteroides melaninogenicus may be significant. However, since collagen is readily denatured at an acid pH
and denatured collagen is susceptible to destruction by several proteases,\textsuperscript{45} this specific enzyme may not be necessary. Fullmer and Gibson\textsuperscript{46} stated that the tissues themselves secrete an active substance (collagenase) against native collagen. Thonard\textsuperscript{47} reports that collagenase is cleared rapidly by the tissues as multiple doses of the enzyme failed to produce measurable tissue changes. Animals with a prolonged challenge did demonstrate a histopathologic change similar to periodontitis. When an immuno-suppressive drug (Imuran) was used with a prolonged challenge of collagenase, little tissue change resulted. Since Imuran kept the immunological activity at a minimal level with no concomitant pathological change, the importance of host factors in tissue breakdown is apparent.
The following table from Schultz-Haudt demonstrates the possible enzyme activities associated with the oral flora.

**ENZYME ACTIVITIES ASSOCIATED WITH MEMBERS OF THE ORAL FLORA**

<table>
<thead>
<tr>
<th>BACTERIA</th>
<th>ENZYME(S) PRODUCED</th>
<th>SUBSTRATE</th>
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</thead>
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<tr>
<td>Staphylococci</td>
<td>Hyaluronidase</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td></td>
<td>Coagulase</td>
<td>Clotting of plasma</td>
</tr>
<tr>
<td></td>
<td>Gelatinase</td>
<td>Gelatine</td>
</tr>
<tr>
<td></td>
<td>Hemolysins</td>
<td>Erythrocytes</td>
</tr>
<tr>
<td>Streptococci</td>
<td>Hyaluronidase</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td></td>
<td>Streptokinase</td>
<td>Fibrin, fibrinogen</td>
</tr>
<tr>
<td></td>
<td>Hemolysins</td>
<td>Erythrocytes</td>
</tr>
<tr>
<td></td>
<td>B-glucuronidase</td>
<td>Glucuronidic linkages</td>
</tr>
<tr>
<td></td>
<td>Proteases</td>
<td>Various proteins</td>
</tr>
<tr>
<td>Diptheroids</td>
<td>Hyaluronidase</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td></td>
<td>Chondroitinase</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>Protease(s)</td>
<td>Various proteins</td>
</tr>
<tr>
<td></td>
<td>Sulphatase</td>
<td>Aryl sulphates</td>
</tr>
<tr>
<td>B. melaninogenicus</td>
<td>Collagenase</td>
<td>Reconstituted collagen</td>
</tr>
<tr>
<td>Spirochetes</td>
<td>Protease(s)</td>
<td>Various proteins</td>
</tr>
<tr>
<td>Gram-negative Cocci</td>
<td>Protease(s)</td>
<td>Various proteins</td>
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</table>
Bacterial flora associated with dental plaque have been shown to produce potential activators of the vaso-active chemicals histamine and bradykinin. The possibility that these activators can participate in the pathological inflammatory response of the gingiva must be considered.

Bacterial antigens, toxins, and proteolytic enzymes are capable of triggering degranulation of the mast cell by activating an enzyme within the cell membrane. The mast cell and the biologically active substances it contains have been extensively reviewed by Zachrisson and Schultz-Haudt. A great deal of speculation has transpired concerning the importance of this cell to inflammation and repair. Selye describes the mast cell as being located at strategic points near small vessels. However, Dienstein, et al., have studied gingival samples and found the mast cell located randomly in the connective tissue. Shelton and Hall have provided histological evidence that mast cell numbers are decreased in chronically inflamed tissue but return to normal levels following resolution of inflammation.

The mast cells are thought to be a major source of histamine, which is released by degranulation. Injury to the mast cell is believed to cause degranulation and expulsion of chemicals which may mediate and govern the process of inflammation. Chemical mediation was first brought to light when Thomas Lewis gave us the classic description of the "triple response." This was the response of normal skin to 'H' substance or histamine and is characterized by a red line, a flare and a
wheal. Since 'H' substance was mainly histamine and the response was similar to the vascular changes in early inflammation, histamine was considered to be the chemical mediator of inflammation.

Menkin was one of the first to observe that the tissue response to histamine and certain serum peptides was different. He attempted to show that the serum polypeptides in the inflammatory exudate would cause the flow of leucocytes from the capillaries, yet would not give rise to the characteristic flare of flushed appearance of the skin as histamine did.

Histamine is rapidly destroyed by tissue histaminases, thus its action is transient. Robbins stated that histamine probably initiates but does not sustain, the inflammatory vascular response. G. P. Lewis has hypothesized that by virtue of histamine's action in increasing vascular permeability, the globulin precursors of a plasma kinin, e.g. bradykinin, could migrate from the blood plasma and become active in the interstitial spaces. Studies have shown four pharmacological actions related to inflammation which can be attributed to bradykinin: vasodilation, increased capillary permeability, accumulation and margination of leucocytes, and the production of pain. Since histamine is rapidly destroyed in tissue it may well be that the longer acting bradykinin will perpetuate the inflammatory vascular response that histamine induced.

Various activators will convert the inactive plasma proenzyme, kallikreinogen, into the active enzyme, kallikrein. This activating enzyme can catalyze the liberation of plasma kinins (bradykinin) from the alpha-2-globulins in the plasma. Among the more prominent activators
for kallikreinogen include proteolytic enzymes, plasmin (streptokinase), activated Hageman factor, and an acid pH. Research has provided evidence of alpha-2-globulins in the sulcular fluid and salivary kallikrein. Kallikrein activators implicated with dental plaque are proteolytic enzymes, streptokinase (an activator of plasmin) and acid pH. It has also been shown that degranulation of the mast cell results in its release of proteolytic enzymes and plasmin.

**LYSOSOMES**

The inflammatory defense mechanism acts by diluting the irritant, walling the area off, removing the irritant by phagocytosis and inactivating the irritant with antibodies. The host cells that destroy the irritant are primarily the leucocytes. Neutrophils are the first of these leucocytes to surround and phagocytize the irritant. The neutrophil contains many granules within its cytoplasm called lysosomes. These lysosomes can act as digestive vacuoles since they are filled with many powerful hydrolytic enzymes. The irritant is inactivated by these lysosomal enzymes after passing through the lysosomal membrane. Autolysis of the neutrophil causes release of these lysosomal enzymes which can lead to definite tissue damage. Taichman has recently demonstrated the effect which an exogenous lysosomal exciting factor can have on the tissues. Repeated daily injections of this exciting factor (filipin) left a chronic nodular lesion that healed very slowly. By rendering the animals leucopenic, the development of this chronic lesion was inhibited. In light of this research it is conceivable that bacterial irritants merely initiate a response that lysosomes may perpetuate for a period of time after the stimuli is removed.
ANTIGEN-ANTIBODY HYPERSENSITIVITY

Hypersensitivity to microorganisms in dental plaque appears to be another etiological factor in gingival inflammation. Certainly, the relatively constant contact of dental plaque to sulcular epithelium would provide an excellent chance for bacterial antigens to diffuse through the epithelium and sensitize the animal. Subsequent antigenic challenge would cause a localized allergic reaction. The presence of gamma-globulins in sulcular fluid and the number of plasma cells in inflamed gingival tissue supports speculation concerning the importance of hypersensitivity in gingival disease.

It may be that the inflammatory process is detrimental rather than advantageous to the character of the periodontal tissues. In a beneficial response to inflammation, the host can overcome the noxious chemicals in the inflammatory fluid. However, with allergic problems, such as are strongly suspected with rheumatoid arthritis, the protection supposedly provided by inflammation may, in fact, become a disease process itself.

Allergic reactions are quite varied and it is not certain that all share similar mechanisms. It is believed that these reactions are associated with specific antigen-antibody reactions which can result in cellular injury. Antigens are generally proteins. However, several polypeptides and polysaccharides can act as complete antigens. An antigen is considered foreign by immunologically competent cells which are then stimulated to produce specialized serum proteins (antibodies) to react specifically with the antigen.
Streptococcal cell-wall particles are antigenic and have the capacity to produce a chronic inflammatory lesion in rabbit connective tissue. The ability of these cell wall fragments to induce the lesion seems dependent on host resistance, susceptibility of cell walls to salivary lysozyme, and production of hyaluronidase. Since streptococci are found in large numbers in dental plaque and can provide antigens for an allergic inflammatory reaction, bacterial antigens from dental plaque may penetrate the crevicular epithelium. Rizzo has repeatedly deposited antigenic protein into healthy rabbit gingival sulci. The lesions that resulted showed accumulations of plasma cells, resembled chronically inflamed tissue, and demonstrated that antigens could cross the epithelial barrier. These experimental results suggest that a gingival condition similar to chronic gingivitis can be elicited by a local absorption of antigens.

ENDOTOXINS

Most gram-negative bacteria have lipopolysaccharide in their cell wall which has a number of physiopathologic effects. These lipopolysaccharides are called endotoxins and are liberated upon autolysis of gram-negative bacteria. Among the physiopathologic effects of endotoxins are: fever production, tolerance, shock and the Shwartzman phenomenon. The Shwartzman phenomenon is a local inflammatory reaction at the initial site of inoculation of endotoxins after a second intravenous provoking dose. It is believed that this reaction is unrelated to antigen-antibody reactions and it remains uncertain whether the Shwartzman phenomenon ever participates in human disease.
Gram-negative bacteria are indigenous to dental plaque, and the early stage of gingival inflammation coincides with an increase in gram-negative bacteria as plaque matures. Therefore, it would be of interest to review the histopathologic effects of endotoxins on the oral mucosa. The work of Mergenhagen has verified that gram-negative oral bacteria contain endotoxins which will elicit the classic Shwartzman reaction. A local injection of endotoxin from an oral Veillonella into rabbit palate demonstrated inflammatory lesions with areas of palatal bone resorption.

Borglum-Jensen, et al., studied human subjects by the "skin window" technique to relate the role of endotoxins in inflammation. They found that the endotoxin from oral bacteria accelerates the migration of leucocytes, and has a stimulating influence on their phagocytic activity.

In a recent experiment using a topically applied endotoxin, Rizzo found that when the sulcular epithelium was intact no absorption into the underlying connective tissue was observed. By ulcerating the sulcular epithelium, the connective tissue did show positive signs of absorption. He suggests that endotoxins may play no role in the initiation of periodontal disease. However, after the disease has progressed to the point that the sulcular epithelium becomes ulcerated, these endotoxins may serve to perpetuate a noxious stimulus to the connective tissue.
CHAPTER III

METHODS AND MATERIALS

Apparatus Design and Adaptation to Experimental Animal

A 25 gauge needle was modified so that its beveled end was adapted to the mesial buccal surface of the Sprague-Dawley rat's maxillary first molar. With this arrangement, experimental solutions can 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 degree angle to the buccal surface. This bend was necessary to accommodate the rat's mandibular movements.

Teflon tubing measuring 0.018 inches in internal diameter was placed over the unbeveled end of the needle to the distal surface of the first molar. 0.012 orthodontic ligature wire was tightly wrapped six times around the tubing and needle in the area adjacent to the buccal surface of the second molar. A 20 gauge trocar was passed subcutaneously from the buccal mucosal surface opposite the third molar and directed posteriorly to be exteriorized in the dorsum of the animal's neck near their midline. The trocar was removed after the free end of the tubing was passed through. The tubing was drawn posteriorly until the free 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 and 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 1)
A diagramatic representation of the apparatus (Figure 2) shows the needle and the tubing tightly secured so that the beveled surface was facing and above, yet not touching the mesial sulcus. Another diagramatic illustration of the apparatus can be seen in Figure 3.

This same procedure was performed on the contralateral side for the control solution.

The exteriorized tubing, before connecting to the perfusion pump, was passed through a plastic pack worn by the animal to prevent his displacement of the tubing. (Figure 4) The teflon tubing, after emerging from the pack, was secured to a vertical metal rod on the pack by tape. Stability was maintained by a small closed coil spring strap connected to the four corners of the plastic pack.

Adjustment of the harness to each individual 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. The spring harness was covered with polyethylene tubing to help protect the animal's skin from irritation. Figure 5 shows the animal wearing the plastic pack and harness.

To provide more protection for the tubing located 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 while allowing reasonable freedom of movement. The superior end of the spring was attached to a horizontal dowel rod supported by a ring stand. The pack and harness were now suspended at a height acceptable to accommodate the rat's movements. Teflon 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 were constructed 9 inches in diameter and 16 inches in height to house the rats individually and aid in unwinding the tubing caused by the animal's circular movements. The free end of the tubing was attached to a compact infusion pump (Model #975, Harvard Apparatus Co., Mills, Massachusetts) capable of delivering solutions at the rate of 0.65 ml/day to 1,152.0 ml/day. (Figure 6)

**Experimental Design**

Male Sprague-Dawley rats weighing an average of 343 grams, previously fed purina chow pellets and water ad libitum, were divided into three groups and fasted during the experimental period. The animals were anesthetized according to body weight (50 mg/Kg) with nembutal intraperitoneally. After the desired anesthesia level was obtained the rats were placed on a rat board to facilitate placement of the apparatus.

In the first group three animals wore the apparatus with the needle coronal to the mesial sulcus of their right maxillary first molar. Their left side remained undisturbed to serve as a control. To observe localization of the solution delivered and any histological change the presence of the needle and ligature wire could induce on the mesial sulcular area of the maxillary right first molar, tap water was perfused at the rate of 4.8 ml/day. This was followed by 2% trypan blue 5 hours prior to sacrifice at the rate of 4.8 ml/day. The animals were sacrificed at 1 day, 4 days and 7 days.
In the second group two rats were perfused with 1% trypsin for 1 day and 4 days at the rate of 4.8 ml/day. The 1% trypsin solution was prepared by dissolving the powdered enzyme, obtained from Sigma Chemical Company, in double de-ionized water. The Trypsin (Bovine-Pancreatic) has an activity of 10,000 BAEE units/mg. The enzyme solution was stored at 4 degrees centigrade and fresh solutions were used every 12-24 hours to prevent inactivation of the enzyme which occurs at room temperature. Double de-ionized water was delivered to the contralateral side for a control.

After completion of enzyme perfusion for 24 hours the first animal was perfused on both sides for 2 hours with 2% trypan blue to observe possible dye penetration in the crevicular epithelium.

The third group of two rats were perfused with 1% pronase at the rate of 0.96 ml/day and 4.8 ml/day for 4 days. A 1% pronase solution was prepared by dissolving the powdered enzyme, obtained from CALBIOCHEM, in 0.02 Molar Tris buffer (2-amino-2 hydroxy-methyl-1,3 propandiol) at pH 7.5. The pronase (Streptomyces griseus) had an activity of 45,000 proteolytic units/gram. The experimental solution was stored at 4 degrees centigrade and fresh solutions were used every 12-24 hours to decrease temperature inactivation of the enzyme. The Tris buffer was delivered on the contralateral side as a control solution.

After the perfusion was completed, the animals were sacrificed with an overdose of nembutal. The animals were weighed, secured to the rat board, and the palates then dissected out.
Histologic Preparation

The palates were placed in 10% Formalin for 24 hours. Demineralization was accomplished by placing the palates in RDO (rapid bone decalcifier from Du Page Kinetic Laboratories) for 8 hours. Following this procedure the palates were embedded in paraffin. Four tissue sections were cut on a microtome in 6 micron sections every 100-200 microns, in a longitudinal plane, to intersect all three molars. The sections were mounted and the first and fourth sections of each 100-200 micron area were stained with hematoxylin and eosin.

Means of Assessing the Apparatus

Localization of the delivered solution was determined by gross observation of the palatal area stained with the dye (trypan blue) from the first group of experimental animals. Any tissue damage the needle and ligature wire could induce on the mesial sulcular area was determined by comparing the histologic sections taken from the control side and the experimental side with the apparatus.

Penetration and localization of the delivered solution into the crevicular epithelium was assessed by examining the unstained sections from the 24 hour trypsin perfusion for dye (trypan blue) location.

Slides stained with hematoxylin and eosin were studied microscopically at 100X and 430X magnification. Observation was directed toward general tissue changes, presence and type of inflammation, and exudate adjacent to the mesial gingival crevice. The stained slides were evaluated by assessing the crevicular epithelium and the subepithelial connective tissue separately. (Table No. 1)
This investigator used a lens which projected a square outline in the center of the microscopic field 200 microns X 200 microns at 430X magnification. Utilizing reference points, this square facilitated recording by localizing the count to a specific area.

The crevicular epithelium was inspected for rete ridge formation, mitotic figures, the numbers of neutrophils and changes in cellular detail. Changes in cellular detail inspected for included: acantholysis, ancanthosis, intra cellular edema, karyorrhexis, karyolysis, and micro-abscesses.

The subepithelial connective tissue was inspected for an area 200 microns from the basal cell layer of the crevicular epithelium for edema, number of neutrophils and number of blood vessels.
CHAPTER IV

RESULTS

Effects of the Apparatus on the Experimental Animal

The animals appeared to adapt reasonably well to their new environment. On recovery from anesthesia, several rats did try to dislodge the tubing emerging from their neck. However, at the termination of all perfusions, needle placement was checked and a relatively constant position was maintained. No irritation due to the friction of the harness was observed. The subcutaneous placement of the tubing produced swelling in the retromandibular area. This area regained its normal appearance after several days.

The fasting animals, perfused from a one to seven day period, lost an average of 71 grams or 20.5% of their original weight. (Table 2)

Localization of the Solution Delivered

A. Gross Observations:

Prior to sacrifice, the animals in group one, (perfused at 4.8 ml/day on one side with tap water followed by trypan blue) were anesthetized with nembutal and the experimental area was observed while the perfusion continued. All animals in group one demonstrated gross localization of the dye perfused to the mesial sulcular area of their maxillary first molar. (Figure 7) The drops of 2% trypan solution emerging from the needle were observed for several minutes. The drops touched the mesial sulcular area and then were dispersed up to the palatal and buccal line angles of the rat's maxillary first molar.
B. Microscopic Observations:

Control side: The dye did not penetrate into the crevice, yet there was some staining of the keratin near the coronal margin of the crevicular epithelium.

Experimental side: The experimental slides showed that the dye actually penetrated the crevicular epithelium. Intracellular and intercellular staining in the crevicular epithelium can be seen in a section from the experimental side. (Figure 8)

Histological Observations (Table 1)

A general histological inspection of the first group of animals (perfused on one side with tap water followed by trypan blue) showed a mild acute inflammation with little difference between the control and experimental 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 cementoenamel junction. A stratum corneum was present to approximately half the distance from the marginal area to the cementoenamel 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 few mitotic figures. Rete ridge formation was seen in both sides of the first animal and in the control sections of the second animal. No changes in cellular detail was observed. No significant difference in the number of neutrophils in the control or experimental sides could be found.

The subepithelial connective examined in the first group demonstrated cells, fibers, vessels and ground substance. The cells were
primarily fibroblasts, a few neutrophils, less lymphocytes, occasional mast cells, plasma cells and macrophages were seen. A small number of vessels were observed with their walls being composed of thin plate-like endothelial cells. Most of this area of connective tissue was comprised of strands of wavy pink staining collagen fibers. Edema, or a wider spacing between the cells and structures within the tissue, was observed on several sections of the animal which was perfused for 7 days.

In the second group of animals, two animals, #4 and #5 received 1% trypsin on the experimental side and double de-ionized water on the control side. The control side which received double de-ionized water revealed a similar histologic picture to that of the animals in the first group. However, both animals had more neutrophils in the connective tissue and animal number 5 demonstrated edema of the subepithelial connective tissue.

The experimental side of animal number 4, (Figure 9) which was perfused with 1% trypsin for one day, at 4.8 ml/day had inflammatory exudate in the crevicular area and a great deal more neutrophils in the epithelium than the control. (Figure 10) Changes in epithelial cellular detail included acantholysis and karyolysis. (Table 1) Also a number of the epithelial cells adjacent to the crevice appeared to be sloughing into the gingival crevice.

The experimental side of animal number 5 (1% trypsin at 4.8 ml/day for 4 days) revealed no significant changes from the control side other than changes in epithelial cellular detail. Some karyolysis and intra-cellular edema was observed. (Table 1)
The control slides from the 1% pronase perfusion, which received the Tris buffer, demonstrated several changes relative to the control slides from group number 1. Slides from rat number 6 showed changes in epithelial cellular detail characterized by karyolysis. In the subepithelial connective of rat number 7 a wider spacing or edema of that area was observed. (Figure 10), (Table 1)

The experimental side of animal number 6 received 1% pronase at the rate of .96 ml/day for 4 days. Epithelial and connective tissue changes were observed. The sections revealed an increase in neutrophils and the epithelial cells adjacent to the enamel appeared to be sloughing with a concomitant widening of the intercellular spaces. Figure 12 shows a dilated capillary projecting into the crevicular epithelium which is filled with neutrophils. Neutrophils are also seen coronal to the vessel in the adjacent epithelium. The epithelial cells adjacent to the capillary demonstrate acantholysis, karyolysis, generalized intracellular edema and loss of cellular detail. (Table 1)

Examination of slides from the experimental area of animal number 7 (1% pronase at 4.8 ml/day for 4 days) revealed inflammatory exudate in the crevicular area which was fibrinoid of nature and contained neutrophils. Intracellular edema of the stratum spinosum, neutrophil infiltration of the epithelium, marked increase in the number of neutrophils in the subepithelial connective tissue and edema of the connective tissue were noted. (Table 1)
CHAPTER V

DISCUSSION

Gingival inflammation involves the interplay of many aggressive local factors and host resistance. Although host resistance is important in gingival disease, it was the purpose of this thesis to describe and evaluate an apparatus capable of delivering the local factors associated with dental plaque and gingival inflammation.

It is apparent, from the literature reviewed, that the bacterial components of dental plaque are the major local factors in the etiology of gingival inflammation. Conclusive evidence has not been found concerning the specific mechanism by which bacterial plaque initiates gingival disease, yet many investigators have speculated upon the role played by many of these bacteria and their products.

Enzyme Selection

The initiation and maintenance of gingival inflammation has many possible pathways. It is conceivable that there is more than one bacterial mechanism propagating inflammation in the gingiva at a given time and that a variety of these mechanisms have a number of potentially pathologic combinations.

Proteolytic enzymes have been associated with the microbial population of dental plaque. Oral Streptococci, Spirochetes, Fusobacteria and gram negative bacteria have been shown to produce proteolytic enzymes which can hydrolyze proteins. The epithelial intercellular substance is
known to contain mucin or glycoprotein. One logical mechanism for initiating gingival inflammation could be that of proteolytic enzymes from dental plaque depolymerizing the intercellular cementing substance between sulcular epithelial cells. This depolymerization may open the door for other noxious chemicals associated with dental plaque and allow them to act on the connective tissue. Once inside the connective tissue proteolytic enzymes also have the capacity to activate the chemical mediators of inflammation.

The hydrolysis of the epithelial intercellular substance would require a fairly powerful proteolytic enzyme. "Epithelial mucins have been considered fairly resistant to the action of common proteolytic enzymes and proteolytic digestion has been used frequently for the isolation and purification of these substances." Proteolytic enzymes that are involved in the hydrolysis of proteins and peptides can be divided into exo- and endopeptidases, according to whether they hydrolyze only the peptide linkages at the end of the polypeptide chain or whether they hydrolyze internal peptide linkages; the latter are undoubtedly more useful from the point of view of tissue disaggregation. Among these, trypsin and pronase are of particular interest because of their high activity and low specificity.

Trypsin is present in the intestinal juices of most animals. It arises from the action of enterokinase upon a precursor, trypsinogen, synthesized in the pancreas. Trypsin was selected for perfusion since it has the capacity to separate epithelial cells. This action has been interpreted as a lysis of a protein containing intercellular substance.
Pronase was selected for perfusion because of its high activity. Pronase is a proteolytic enzyme from the *Streptomyces griseus* and has been found to cause extensive degradation of mucin which produced considerable amounts of free amino acids. Weinstein has demonstrated the ability of pronase to cause a rapid epithelial cellular detachment in the absence of any significant effects on viability or on chromosome morphology. Bauer has shown that pronase is more effective in removing the periodontal ligament fibers for allogenic tooth transplants when compared to collagenase and hyaluronidase.

**Perfusion Parameters**

The length of perfusion of 1 day and 4 days was used in order to study the early histological changes induced by the proteolytic enzymes. The rate of perfusion at 4.8 ml/day was determined by preliminary studies which revealed that faster rates, on this experimental animal, complicated localization of the delivered solution. With a slower delivery rate, evaporation and interference from the rat's tongue could theoretically reduce significantly the amount of solution to reach the experimental area.

A concentration of 1% was used because previous experimentation has shown tissue change at this concentration. Another reason for using low enzyme concentration was that trypsin has a high catalytic center activity. This means that the actual number of trypsin molecules necessary to attack a given number of substrate molecules would be much less than for an enzyme with less catalytic center activity.
**Animal Response**

The animals appeared to adapt reasonably well to their new environment. After one day they appeared alert and moved around in their cages. Occasionally a rat would climb to the top of the cage. This happened infrequently and presented no problem.

The animals were fasted in order to prevent mastication of food from disturbing the apparatus. Stahl has shown that rats which were wounded and simultaneously placed on a protein free diet showed a delay in connective tissue repair. The tissue effect, on the animals in this experiment, would be difficult to determine due to the nutritional deprivation and the stress created concomitant with the environmental changes. However, it can be stated that microscopic observations of the mesial sulcular area showed little change between animals of the first group which were perfused with water and dye on one side compared to the opposite side which was undisturbed.

**Histological Evaluation**

In order to summarize and illustrate histological findings a chart was designed to record specific tissue changes. (Table 1) The rationale for selecting these tissue changes for inspection was as follows:

1. The classic anatomical description of early inflammation includes edema, exudate, accumulation of leucocytes and vascular dilation.49

2. Marwah has demonstrated that the number of mitotic figures in the epithelium increased markedly when associated with gingival inflammation.
3. Grant and Orban have demonstrated rete ridge formation in the culcular epithelium from inflamed gingival specimens. The absence of this rete ridge formation has been generally accepted as "normal" on non-inflamed gingival specimens.

Histological examination of the first group of animals showed a similar inflammatory picture on both control and experimental sides. However, some slight differences were noted. The experimental side of animals #2 and #3 did show edema in the connective tissue. Whether this edema was due to irritation from tap water or dye or trauma from the needle is unknown. Rete ridge formation correlated positively with a higher number of neutrophils in the epithelium on the control and experimental sides. These minor differences noted between the control and experimental areas indicate that the presence of the interproximal wire and the perfusion needle had little effect on the experimental area.

Histological observation of the second group of animals revealed that the experimental area of animal #4 (perfused with 1% trypsin at 4.8 ml/day for 1 day) had the most marked epithelial changes of any animal examined. A possible explanation for this could have been a lowered host resistance for this particular animal.

Perfusion of animal #4 with trypan blue 2 hours prior to sacrifice did show penetration of the dye in the crevicular epithelium on the experimental side. This lends support to the assumption that the experimental solution was, in fact, gaining entrance into the gingival crevice.

Histological sections examined from the two rats which received the 1% pronase perfusion showed differences between control and experimental
areas. More epithelial changes in cellular detail and more neutrophils in the epithelium were found on the experimental side. Animal #7 which received pronase at a faster rate (4.8 ml/day had more marked changes compared to animal #6 (1% pronase at .96 ml/day). Sections from animal #7 demonstrated a fibrinous exudate with neutrophils adjacent and coronal to the crevicular epithelium. More neutrophils were found in the sub-adjacent connective tissue. (Table 1) These results could imply that a faster delivery rate produced more tissue changes.

**Comparison With Other Enzyme Investigations**

Intermittent injection of enzymes\(^{32,40,47}\) for a considerable period of time has produced connective tissue changes. The work of De Vincenzo and Jefferies\(^{89}\) demonstrated a continuous in vivo perfusion apparatus which produced obvious tissue changes with less concentration of experimental enzymes over a brief period of time. Their results may indicate the importance of continuous rather than intermittent application of enzymes.

However, their apparatus had several deficiencies. Great care and meticulous attention had to be given to the apparatus during the time of perfusion. For ideal function an investigator had to be near at hand during the perfusion, thereby making perfusion of longer than 24 hours very difficult. Consistent and reproducible perfusion rates were difficult to maintain. Reloading and recharging the system with butane gas had to be done every 4-6 hours. This required removing the animal from his cage at these intervals and could result in a changed position of the needle.
A major objection to their work and the previously mentioned studies involving enzymatic injections could be based on traumatic entry of the enzymes. The trauma introduced by needle placement could not be avoided and the interaction between trauma and exogenous factors complicated evaluation of tissue changes.

The apparatus described in this report has none of these limitations. The results of this experiment have shown that the placement of the needle is atraumatic, that the experimental solution delivered is localized, and that slight inflammatory and epithelial changes could be induced on the experimental area with non specific proteolytic enzymes.

Atraumatic intermittent application has produced gingival effects which have been less than dramatic. The tissue changes described in this report were minimal and should be given a reserved significance. It was hypothesized that with the continuous delivery of powerful proteolytic enzymes more dramatic gingival changes should have been noted. In a more ideal experimental situation the epithelial cellular changes and the increased number of neutrophils in the crevicular epithelium in the experimental areas could be interpreted as a reaction to the presence of diffusable proteolytic enzymes. Since marked changes were not noted, several possible reasons for the mild changes observed should be discussed.

Grant and Orban have stated that the "epithelial attachment" may serve as a pathway for the diffusion of bacterial products to the subadjacent lamina propria. They have found a positive correlation between the presence of neutrophils clustered in the sulcular epithelium and
calculus on the adjacent tooth surfaces in histological sections taken from humans. The presence of bacterial plaque and calculus on the teeth of the albino rats in this experiment could have precluded the effects of the experimental enzyme perfusion.

The rat has an innate resistance to periodontal disease and has an intact keratinized crevicular epithelium which may prevent penetrability of the area. Keratin is an abluminoid (a tough type of protein) which is relatively insoluble and impermeable. It forms a protective sheet which preserves the fluid environment of the deeper layers of cells.

Although the biuret test was performed to determine and confirm that the freshly prepared proteolytic solutions did contain noticeable activity, the proteolytic activity of the solution was not checked after delivery to the experimental area. Inactivation may well have occurred while the enzymatic solution was being delivered by the Harvard perfusion pump. Temperatures as high as 42°C were recorded inside the control syringes placed on the running pump.

Since all enzymes are proteins, the possibility exists that the powerful non specific proteolytic enzymes used in this experiment were self digesting and the time of activity limited. This may account for the lack of significant tissue differences between the 1 and 4 day trypsin perfusions.

Suggestions for Future Studies

The slight tissue changes produced with the powerful enzymes used in this perfusion could also be interpreted to indicate that future use of the apparatus should be modified.
Perhaps, a better experimental animal for the application of this apparatus would be a rabbit.\textsuperscript{42} This animal has non-keratinized crevicular epithelium similar to that found in humans, normally a low degree of inflammation in the gingival tissues and a larger oral cavity which would allow localization of experimental solution at a faster delivery rate.

Other modifications which could make the results more meaningful include: labelling of the enzyme to document its penetration, intravascular injection of trypan blue prior to sacrifice to demonstrate changes in vascular permeability, better control of enzyme temperature and more frequent changing of enzymatic solutions to prevent possible inactivation or autolysis.
CHAPTER VI

SUMMARY AND CONCLUSIONS

This thesis describes an in vivo perfusion apparatus which can continuously deliver controlled quantities of solutions to localized, contralateral intra oral areas in albino rats. The animals were anesthetized. A 20 gauge trocar was used to pierce the epidermis in the scapular region and then directed subcutaneously to pierce the buccal mucosa opposite the third molars. Small teflon tubing, attached to a contoured 25 gauge needle with a 90 degree bend near its free end, was passed through this trocar. The needle, positioned so that its free end was touching the mesial surface of the first molar was securely fastened by 0.010 gauge ligature wire around the second molar. The trocar was then removed and the procedure repeated on the opposite side. The teflon tubes exit from the dorsum of the animal and pass through a plastic harness which is worn by the animal. The harness prevents the animal from disrupting the teflon tubes which pass upward for two feet before being connected to the pump. A Harvard perfusion pump with delivery rates from 0.65 ml/hr. to 1,152.0 ml/hr. was used. The animals were housed in a freely rotating circular cage and were relatively unrestrained during perfusion.

Localization of the delivered experimental solution was demonstrated grossly and histologically with trypan blue. Histological examination has consistently shown the intra oral needle placement was atraumatic to the
localized area of perfusion. Solutions of 1% trypsin and 1% pronase have been perfused on the experimental side while the appropriate control solutions were used on the opposite side. The results obtained have shown that a continuous flow of non specific proteolytic enzymes can elicit a moderate gingival inflammatory response in the albino rat. Histological examination revealed little inflammatory change in the control.

The information provided by this thesis suggests that with modification the described apparatus can be useful in studies on gingival disease. The apparatus itself has a great deal of research potential. 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.

It would seem logical, in view of the limitations in our present knowledge, for investigators to continue to probe into the relationship of the inflammatory potential of the bacterial components of dental plaque. Hopefully, with the availability and intelligent use of more sophisticated techniques, further research will provide a clearer picture of the etiology of gingival disease.
BIBLIOGRAPHY


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0 = Absent
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TABLE 2. A SUMMARY OF THE EXPERIMENTAL PROTOCOL AND THE WEIGHT CHANGES

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**Perfusion on Mesial Sulcus**
- Tap water and Trypan (1) day at 4.8 ml/hr.
- Tap water and Trypan (4) days at 4.8 ml/hr.
- Tap water and Trypan (7) days at 4.8 ml/hr.
- 1% Trypsin perfusion (1) day at 4.8 ml/day followed by Trypan for 2 hours.
- 1% Trypsin (4) days at 4.8 ml/day
- 1% Pronase (4) days at .96 ml/day
- 1% Pronase (4) days at 4.8 ml/day

**Animal Number**
- 1
- 2
- 3
- 4
- 5
- 6
- 7
LOMA LINDA UNIVERSITY
Graduate School

AN IN VIVO PERFUSION APPARATUS FOR STUDIES ON GINGIVAL DISEASE:
ASSESSMENT BY A PERFUSION OF NON SPECIFIC PROTEOLYTIC ENZYMES

By
Don Dornan

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

June 1969
This thesis describes an in vivo perfusion apparatus which can continuously deliver controlled quantities of solutions to localized, contralateral intra oral 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. A 1% solution of trypsin was perfused on the experimental area of one animal for one day at the rate of 4.8 ml/day and on another animal for four days at the same rate. A 1% pronase solution was perfused for four days on one animal at the rate of .96 ml/day and another animal at 4.8 ml/day. The appropriate control solutions were delivered on the contralateral side of each animal undergoing enzyme perfusion. Data obtained indicated moderate inflammatory changes were noted on the side receiving the enzyme. More meaningful results should be obtained with the modifications discussed in this report.