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A Study of Protease Activity of the Human Gingiva

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A STUDY OF PROTEASE ACTIVITY OF THE HUMAN GINGIVA

by

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LIFE

Louis M. Orzolek, the younger of two children, was born January 24, 1934, in Kenosha, Wisconsin.

He was graduated from Saint Catherine High School Racine, Wisconsin in June 1951. From September 1951 to June 1953 he attended Marquette University College of Liberal Arts.

In September of 1953 he began studies at Marquette University School of Dentistry and received the Doctor of Dental Surgery degree in June 1957.

He entered the Armed Services of the United States as a first lieutenant in the United States Air Force. In July 1957 he was assigned a rotating dental internship and interned at Lackland Air Force Base Hospital at San Antonio, Texas from July 1957 to July 1958. He was then stationed at Lowry Air Force Base, Colorado from October 1958 to August 1960. He served in the capacity of base periodontist during the last year of this tour.

In September 1960 he began his graduate studies in the Department of Oral Biology of Loyola University.

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CHAPTER I

INTRODUCTION

AND

STATEMENT OF THE PROBLEM

Investigators in recent years have shown more and more the importance of enzymes in bodily processes. Receiving equal attention of investigators are the enzyme inhibitors. Since the beginning of the twentieth century it has been known that animal and human sera have an anti-proteolytic enzyme action. This phenomenon has been investigated in relation to many diseased states, among which are included those with a common ground of severe inflammatory activity or tissue necrosis. The importance of certain enzyme activities in inflammation has also been established. Investigations have been performed which show the presence of proteolytic enzyme inhibitors in animal and human tissues. Ultimately, it would seem that the proper functioning of bodily processes is greatly dependent upon the interrelationship of enzymes and their inhibitors.

The purpose of this investigation was to study the activity of proteolytic enzymes in human gingival tissue under normal and in chronically inflamed conditions in light of the available information regarding proteolytic enzyme inhibitors.

Since little methodology in respect to this type of investigation is present in the literature, the development of a workable technic is considered to be an important part of this study.

CHAPTER II

REVIEW OF THE LITERATURE

Studies of the antiproteolytic activity of tissues have been preceded by the investigation of the antiproteolytic activity of the serum of animals and humans. This field of investigation is closely related to the problem of this paper and a review of the literature of the antiproteolytic action of the serum will provide a fuller understanding of the present problem under investigation.

Hildebrandt (1893), Camus and Gley (1897) and Landsteiner (1900) have been credited by other reviewers of the literature for the first observations of the inhibitory power of serum on the action of trypsin. However, review of these publications shows that the experimental technic involved in these works was primarily the injection of an enzyme, which is protein in nature, into experimental animals. The reactions observed were inhibitory to be sure, but must be interpreted as an antibody reaction to the protein nature of the enzyme. These works, therefore, do not demonstrate the presence of an enzyme-antienzyme reaction with which this paper is concerned as stated in the first chapter.

Opie (1905) studied the enzymatic and antienzymatic action of inflammatory exudates, by production of experimental inflammatory reactions in the peritoneal cavities of experimental animals. By collecting the leukocytes produced in the inflammatory process and allowing the autolysis of these cells, he determined the proteolytic enzyme activity of

the leukocytes. Opie was able to demonstrate the inhibitory effect of blood serum and inflammatory exudates upon the enzyme leukoprotease of the leukocytes by incubation of these with the leukocytes. From this the conclusion was made that serum had the power of inhibiting proteolytic enzymes of the leukocytes and that this inhibitor probably passed into the exudates of the inflammatory process. In the later stages of inflammation there was some diminution of the antienzymatic action.

Hussey and Northrop (1922) investigated the antitryptic activity of the blood. They found that the inhibitory agent and trypsin combine to form an inactive but dissociable compound. The reaction in equilibrium could be expressed by the equation:

$$\text{Trypsin} + \text{inhibitor} \rightleftharpoons \text{trypsin·inhibitor}$$

These investigators stated that the conditions of equilibrium are governed by the law of mass action.

Kunitz and Northrop (1936) developed a method of crystallization of a trypsin inhibitor from beef pancreas. The inhibitor also inhibited chymotrypsin but to a lesser extent. The inhibitor displayed the general properties of a polypeptide.

Grob (1943) studied the nature and experimental variation of the antiproteolytic activity of serum by intravenous, intramuscular and oral administration of trypsin in rabbits. He found that intramuscular administration of trypsin resulted in a slow rise in the antiproteolytic activity of the serum followed by a lesser decline. Intravenous administration resulted in no appreciable variation. Oral administration resulted in a

rapid rise, which was sustained during the period of administration. Intramuscular, intravenous and oral administration of denatured trypsin resulted in no appreciable variation.

Determinations of antiproteolytic activity of the serum were made by the degradation of casein and by a proposed experimental formula devised by author. From these facts and from the findings of Kunitz and Northrop, mentioned above, Grob attached important significance to the products of protein hydrolysis both in the intestine and parenterally in relation to the antiproteolytic activity of the serum. On the basis of the nature and magnitude of the experimental variations obtained in his work and on the lack of parallelism between precipitin tests performed on the animals and antiprotease activity, Grob stated that antibodies to trypsin were not an important factor in the antiproteolytic activity of the serum.

Investigating further the nature of the antiproteolytic substance in the serum, Grob (1945-46) presented evidence that leukoprotease and trypsin are inhibited by reducing agents, including thiol-sulfhydryl compounds and ascorbic acid. It was shown that the antiproteolytic activity was largely confined to the albumin fraction of serum. According to Grob the inhibitory factor was not dialyzable. The reported fact that the antiproteolytic factor was not freely diffusible in the body except when inflammatory or other pathologic processes increase capillary permeability, is in agreement with the conclusion of Opie.

Dutherie and Laurenz (1949) supported the findings that the serum antiprotease is associated with all the serum protein fractions but main-

ly with the most soluble albumin fraction. They also found that the serum trypsin inhibitor was relatively stable but was sensitive to the lowering of pH, which is in agreement with the findings of Grob (1945). Dutherie and Lorenz were unable to reproduce the results of Grob concerning the variations of the antitryptic levels of serum, by the administration of trypsin. In their studies there was only a 15% increase in the anti-trypsin activity of serum after four weeks of subcutaneous administration in rabbits. No increase was observed after oral administration.

Rusk and Cliffton (1951) investigated the relationship of the anti-proteolytic activity of serum with the fibrinolytic activity. By injecting soybean trypsin inhibitor of Kunitz and Northrop intravenously into mice they were able to increase the antitryptic activity of the serum. The changes were observed for a week or longer following the injections, indicating the slow destruction or excretion of the soybean inhibitor in vivo.

The relation of a polypeptide structure to the antiproteolytic factor in serum has been reported often in the literature. Ungar and Adler (1956) have reported the discovery of a peptidase in guinea pig serum whose activity closely parallels the guinea pig serum protease inhibition. The action of the peptidase is detectable by its action on a chromogenic substrate glycyl-beta-naphthylamine. Competitive inhibition of the peptidase action by both plasmin and trypsin suggested that the proteases were substrates for the peptidase and that the antiproteolytic activity of guinea pig serum was due, at least in part, to the enzymatic destruction of proteases. While this phenomenon was found to be present in

other species including man, its relationship to protease inhibition was not as marked.

In discussing proteolytic enzyme inhibitors John Bodman (1958) specifies the presence of two separate inhibitors for trypsin and chymotrypsin. According to Bodman both could be separated by curtain electrophoresis. The trypsin inhibitor appeared in several fractions and the chymotrypsin was always collected in the fraction between the α_1 globulins and the albumin. Bodman suggested that the serum inhibitor titers might possibly be under hormonal control.

Aizawa (1960) conducted zone electrophoresis studies to determine the relationship of trypsin and chymotrypsin inhibitors to each other. Using a newly developed zone electrophoresis technic it was determined that the greater part of both trypsin and chymotrypsin inhibitors migrated with the alpha globulin fraction. Some inhibitory activity was found with the α_2 globulin. Studies of the pathological sera showed the same distribution of the inhibitors in the α_1 globulin. The ratio of inhibitory activity between trypsin and chymotrypsin inhibitors in normal and pathological sera did not vary significantly. As a result of these findings it was concluded that the inhibitory activity against trypsin and chymotrypsin was exerted by a single inhibitory substance.

Vermeenko (1960) found that serum and plasma inhibited trypsin activity to the same degree and whole blood was 30% less inhibitory. When casein was used as a substrate, the trypsin activity inhibition depended upon the amount of plasma. The addition of urea to casein substrate caused

a marked deviation from the linear dependence. Curves representing trypsin activity dependence pointed to the dissociation of the trypsin inhibitor complex. The effect of urea on the complex dissociation indicated the involvement of H bonds in the mechanism of the trypsin inhibitor complex. The dependence of the dissociation of the trypsin inhibitor complex on the nature of the substrate showed that the substrates were capable of competing with the inhibitor for the free trypsin. A dynamic equilibrium established itself between the trypsin and the inhibitor on the one hand and the substrate on the other.

Thus far the presence of an antiproteolytic factor in the serum has been established and a beginning insight into its nature has been discussed. However, the problem of this work concerns the presence of a proteolytic enzyme inhibitor in tissue. Opie (1905) discusses the possibility of the passage of the serum inhibitors into tissues in inflammatory exudates. This possibility has been confirmed in the literature. However, the investigations of a tissue inhibitor have been undertaken only recently and to a limited extent.

Astrup (1952) described a potent fibrinolytic inhibitor in ox lung tissue which appeared to be an antiproteolytic substance.

Scevola, Calchi, Novati, and Felisati (1954) and Scevola and Felisati (1955) investigated human, rabbit, guinea pig and ox tissues which included lung, heart, liver, kidney, muscle, stomach, uterus and placenta. The studies were done on homogenates of these tissues. Trypsin inhibition varied between tissues of the same animal and between species for

the identical tissue. Upon 20 minute contact trypsin inhibition by lung was 90, 78, 35, 29 and 28% in ox, guinea pig, humans, rat and rabbit respectively. The inhibition for heart tissue was 50, 42, 38, 34, and 11% for guinea pig, rat, ox, rabbit and humans; for placenta 67, 63, 26, 18 and 11% for humans, guinea pig, ox, rat, and rabbit respectively. Guinea pig uterus showed maximum inhibition; low inhibition was shown for muscle, stomach, kidney and liver tissues. In general, chymotrypsin was inhibited much less than trypsin. Human tumoral tissue showed an irregular behavior. The antitryptic activity was sometimes lower, as in lung, and sometimes higher, as in brain, than in normal tissue.

Astrup and Stage (1956) isolated and partially purified a protease inhibitor from ox lung tissue. The active compound had a characteristic thermostability and was most stable at acid reaction and was destroyed at neutral and alkaline reaction. These pH reactions are the reverse of those of the serum antiproteolytic factor reported this far.

By subjecting tissue monocytes in a culture to an antigen-antibody reaction, Tokuda (1959) studied the proteolytic mechanism of the antigen antibody reaction. Sensitized rabbit monocytes were subjected to an antigen-antibody reaction in a specially designed culture chamber and were found to release a protease into the culture fluid. Beginning at 24 hours and up to 48 hours a proteolytic inhibitor was released. Evidence was given that the inhibitor was definitely of cellular origin. The protease inhibitor was isolated and partially purified and found to also inhibit the protease of euglobin fractions prepared from the area of arthus-like

hypersensitivity and papain but not of trypsin. It was pointed out that both of these proteases had an essential SH group. The inhibitor also gave a slight Biuret reaction indicating that it might have been a polypeptide. Although this study was essentially concerned with the antigen-antibody reaction of cells, viewed in the light of responses to cellular injury, it is particularly interesting and may be related to the problem studied in this paper.

The relationship of the antiproteolytic activity of the serum and various disease states has been confirmed in the literature. One of the earliest works, that of Opie (1905), implicated the antiproteolytic factor with the inflammatory process. However, one of the earliest investigations correlating the antiproteolytic activity of serum and the inflammatory process was that of Hort (1909). He noted that the inhibitory serum levels were elevated in all the cases of infectious diseases studied. The levels remained elevated through the course of infection until convalescence had been fully established. Antitryptic levels of serum were also elevated in pathological cases involving cellular destruction which were not of infectious causes.

An examination of 2,000 cases by West and Hilliard (1949) indicated a relationship of proteolytic enzyme inhibition, specifically chymotrypsin and rennin, to numerous disease states. In this study acute infectious conditions, influencing chymotrypsin inhibitors predominantly, comprised 24% of all of the cases studied.

The work of Dillard and Chanutin (1949) established some facts which

should be kept in mind. This work studied serum levels of proteolysin, plasmin, and trypsin inhibitor of plasma in cancer patients and a variety of acute and chronic disease states. No relationship between all of these was evident in the study. However, both proteolysin and trypsin inhibitor could be related to cancer and to acute infectious disease states as evidenced by increased serum level. The trypsin inhibitor concentration level remained elevated in those who had been operated on and in those whose tissues were undergoing readjustment to the normal state.

Tauber (1950) found that of cases examined 8.1% of those with acute infections showed abnormal tests of chymotrypsin inhibition. Eight and two tenths per cent of those with chronic infections showed abnormal levels.

CHAPTER III

MATERIALS AND METHODS

This study was performed upon sixteen subjects of whom nine served as a control group and seven as an experimental group. It was decided to limit the study to male subjects in an age range from 25 to 35 years. This was done to eliminate as much as possible variations resulting from sex differences and variations which might be attributable to aging processes. The medical histories of both the control and the experimental subjects revealed no recent systemic diseases of consequence. The control subjects were apparently healthy at the time of the study. The experimental subjects were healthy aside from the periodontal disease.

The tissue studied was the attached gingiva. Tissue specimens were obtained from the vestibular and interdental aspects of the pre-molar or anterior teeth of either the maxilla or mandible. The decision as to which area was to be biopsied was based upon clinical diagnosis. The areas biopsied in the subjects of the control or normal group show clinically normal gingival tissues. The areas taken from the subjects in the experimental group showed chronic inflammation. An histological specimen, stained with hematoxylin and eosin, was made of each tissue sample to confirm the clinical diagnosis of the state of the tissue.

Orban periodontal knives were used to excise the tissue specimens. Anesthesia was obtained through infiltration of procaine hydrochloride anesthetic with epinephrine 1:50,000 concentration. The injection was made as far removed as possible from the sight of the operation. The

specimen was washed in tap water and blotted dry. At this time a portion was removed from this specimen, fixed in 10% formalin and prepared for histologic examination as mentioned above. The histological diagnosis of the state of the tissue was determined by an histologist other than the present investigator. The greater portion of the specimen was placed into a Petri dish, covered and placed in a freezer compartment at about -20 degreed Centigrade. Proteolytic activity of the specimen was determined within the next twenty-four hours as will be described below. In most instances, a sample of venous blood was also obtained in order to assess the proteolytic behavior of the serum associated with the gingival specimens.

The method of Bodman (1957) for the measurement of the proteolytic enzyme inhibitor of serum was adapted to this study. The principle involved in this method is to establish an enzyme:substrate model of known concentration and reaction rate. This model served as a control. To determine whether tissue contained substances which affect the rate of this enzymic activity, an homogenized tissue specimen of known weight and concentration was allowed to incubate in contact with the determined amount of enzyme for a given period of time. This modified enzyme was then incorporated into an enzyme:substrate model. The amount of hydrolysis of the substrate by the enzyme was determined for both the control model and the experimental model whose enzyme constituent had been allowed to be in contact with the homogenized tissue before acting upon the substrate.

Reagents for an enzyme:substrate model were prepared in the following manner. Eight mg. of vitamin free test casein code number CMX-1155

contributed by Abbott Laboratories were added to 180 ml. of distilled water with 6.0 ml. of 1N sodium hydroxide. This was shaken in a stoppered wide-mouth flask twenty times and allowed to stand for one hour at room temperature. To this emulsion was added 284 ml. of distilled water, a crystal of thymol and a few drops of octyl alcohol. This solution is both the substrate and the buffer of the system. The final pH is 7.6.

The enzyme used for this study was chymotrypsin. Chymotrypsin was chosen as the proteolytic enzyme because of the available knowledge about it and because of the reliability of its reaction. Seventy mg. of crystallized chymotrypsin (1075 Armour Hb units/mgm.) contributed by Armour Pharmaceutical Company were diluted in 250 ml. of 0.002 M hydrochloric acid. Then 65.3 ml. of this enzyme solution were diluted to 100 ml. with 0.002 M hydrochloric acid. This was termed the stock enzyme solution. It could also be prepared by weighing 18.3 mgm. of chymotrypsin and diluting it to 100 ml. with 0.002 M hydrochloric acid. Hydrolysis studies of the chymotrypsin with casein demonstrated that further dilution of the stock enzyme solution was necessary in this study, because of the great activity of the enzyme supplied to us. A ten-fold dilution was decided upon. It was prepared by diluting 1 ml. of stock enzyme solution to 10 ml. with 0.002 M hydrochloric acid. This was then called the working enzyme solution. Hydrolysis rates were determined and hydrolysis curves were plotted. From these the enzyme:substrate model concentrations used in this study were determined. Aliquots of the standard enzyme and working enzyme were made according to the protocol shown in Table I. Each

TABLE I

CHYMOTRYPSIN HYDROLYSIS STUDY

TABLE I

INCUBATE 2 HOURS AT 38° C.					MIX LET STAND CENTRIFUGE			SHAKE INCUBATE CENTRIFUGE DECANT		% TRANSMISSION	
Tube	Enzyme	HCL	Saline	Casein	TCA 10%	Digest		NaOH	$CuSO_4$	Trial 1	Trial 2
1	5.0	2.5	2.5	15	3	5		1	1	12.0	9.0
2	2.5	5.0	2.5	15	3	5	PIPETTE	1	1	14.5	12.0
3	1.0	6.5	2.5	15	3	5		1	1	17.0	18.5
1 : 10											
4	7.5	0	2.5	15	3	5	5 ml.	1	1	20.5	21.5
5	5.0	2.5	2.5	15	3	5		1	1	24.0	25.8
6	2.5	5.0	2.5	15	3	5	SUPER-	1	1	32.0	32.0
7	1.0	6.5	2.5	15	3	5	NATANT	1	1	47.0	48.5
8	0	7.5	2.5	15	3	5		1	1	83.0	86.0
←---------- HYDROLYSIS ----------→					←STOP REACTION→			←---------- BIURET REACTION ----------→			

aliquot was made up to 10 ml. volume as shown. Fifteen ml. of casein solution, prepared as described above, were added to each test tube. These tubes were then placed in a water bath at 38 degrees Centigrade for two hours.

The degree of hydrolysis was determined by measuring the quantity of protein hydrolysis products produced. A set of serially numbered tubes, each containing 3 ml. of 10% trichloracetic acid was prepared. Five ml. of the contents of the respective tubes containing the digestion mixture were added to the prepared trichloracetic acid-containing tubes. The tubes were mixed by inversion and allowed to stand at room temperature for 10 minutes. This procedure stopped the hydrolysis reaction of the enzyme and substrate. The tubes were then centrifuged at 3,500 r.p.m. for 20 minutes. The supernatant was pipetted into another set of identically numbered tubes. The products of hydrolysis were then measured by the Biuret method.

The trichloracetic acid soluble material in the supernatant liquid represented the products of hydrolysis. The percentage of trichloracetic acid that is used in this procedure is critical. The trichloracetic acid soluble material consists of polypeptide chains of a particular molecular weight which are the result of chymotrypsin action upon the protein, casein. Greater concentrations of trichloracetic acid would precipitate not only whole protein molecules not yet digested but also a portion of the polypeptide chains as well. Lesser concentrations of trichloracetic acid would fail to precipitate intact molecules of polypeptides. Therefore,

variations in the trichloracetic acid concentrations would influence subsequent analysis of the supernatant liquid.

The supernatant liquid was prepared for the Biuret reaction by pipetting as much of the supernatant as possible into correspondingly numbered tubes containing 1 ml. of 30% Sodium hydroxide. To these tubes 1 ml. of 5% w/v copper sulphate was added and the tubes were shaken to break up the gel which formed. The tubes were then placed into a water bath at 38 degrees Centigrade for 10 minutes after which they were again centrifuged for 10 minutes at 3,500 r.p.m. The Biuret color thus developed was measured by means of a Coleman Junior spectrophotometer. The filter was set at 605 λ. The results were recorded in % transmission and were charted on graph paper.

The resulting graphs are shown in figures 1 and 2. From this the concentrations for the enzyme:substrate model were determined and set at 2.5 ml. of the so-called working enzyme solution and 15 ml. of casein prepared as described above.

Sample tissue specimens obtained in the manner described above and stored at -20 degrees Centigrade were used to determine the proper concentration of the tissue homogenate to be used. The tissue was weighed on an accurate laboratory balance to the nearest tenth of a milligram. The tissue was then homogenized in a Potter homogenizer, the pestle being fitted by a friction clutch to an electric motor. The homogenizer was submerged in a melting ice water bath during the entire time of homogenation. The trial specimens weighed 54.5 mg. and 61.9 mg. These

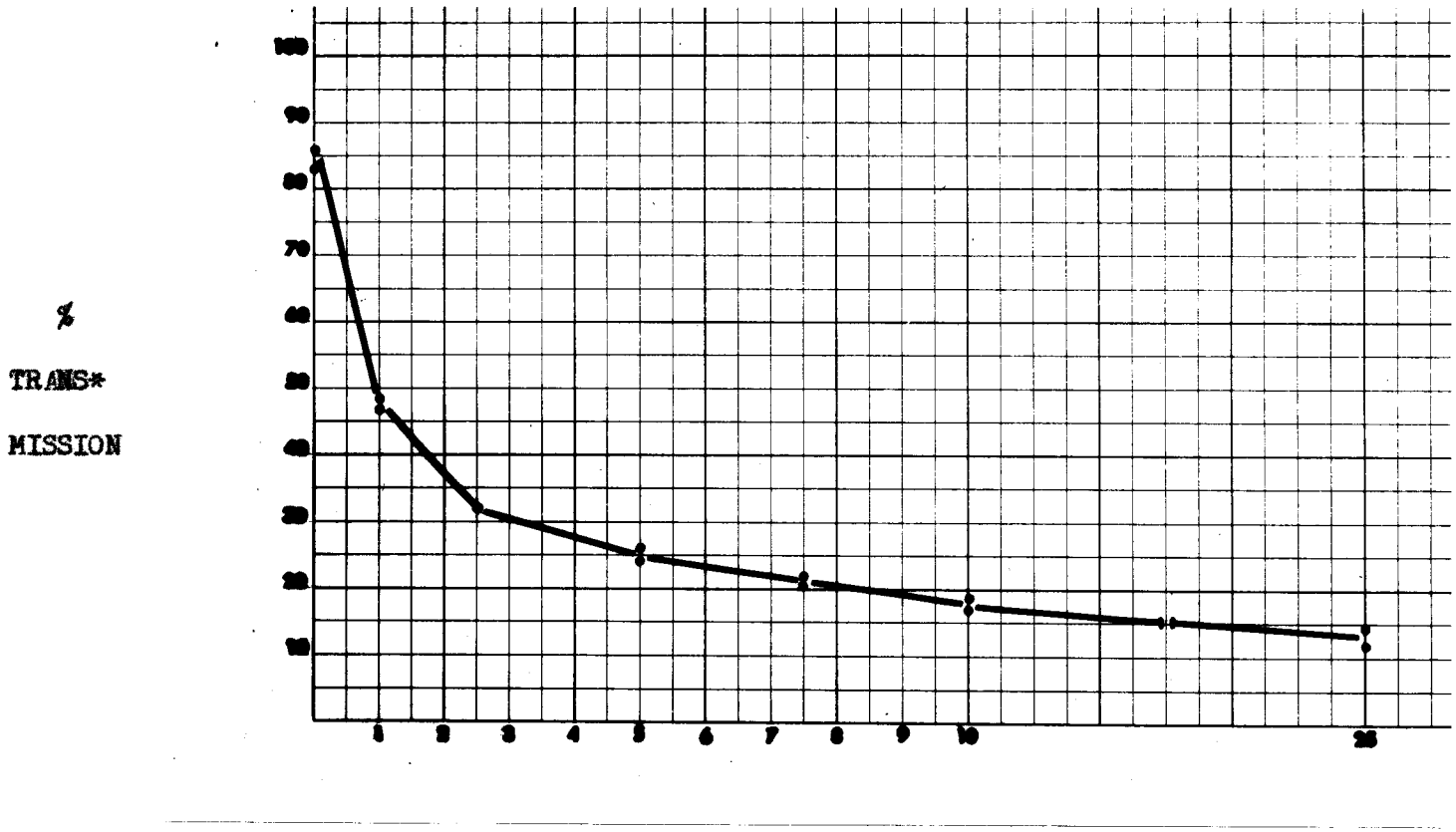

FIGURE 1.

GRAPHIC RESULTS OF HYDROLYSIS STUDY

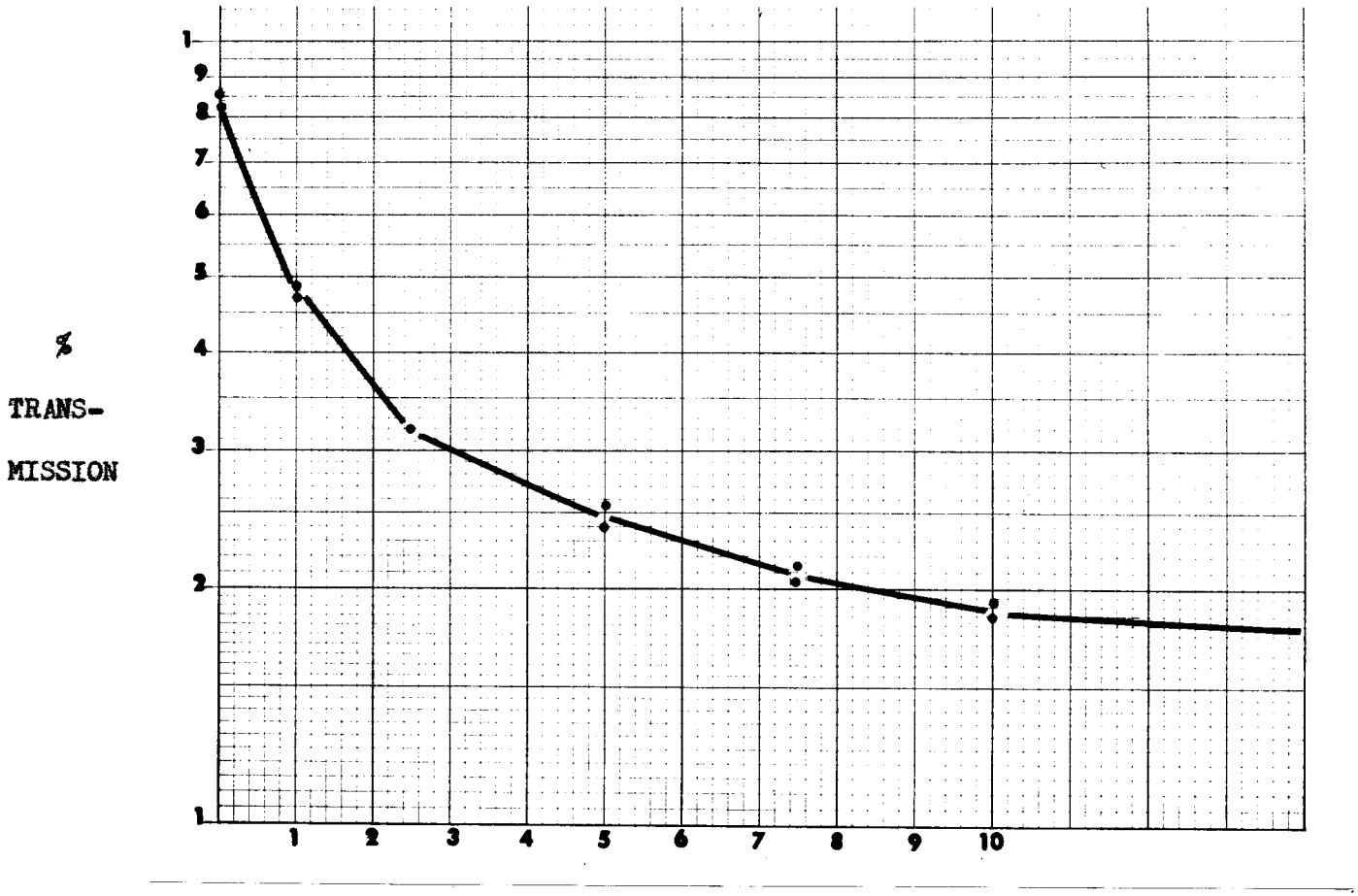

FIGURE 2.

GRAPHIC RESULTS OF HYDROLYSIS STUDY

were homogenized in 15 ml. and 17 ml. of saline solution in order to have identical concentrations. Aliquots of the tissue homogenates were made as shown in Table II. Two and one-half ml. of the working enzyme were added to each of the aliquots and allowed to stand at room temperature for twenty minutes. Each tube was agitated once each minute. Fifteen ml. of casein were then added to each aliquot and the tubes were incubated for two hours at 38 degrees Centigrade. Each tube was agitated every five minutes. The reaction was then stopped by the addition of 3 ml. of 10% trichloracetic acid. The sample was prepared for the Biuret reaction as described above. The protocol for this procedure and the spectrophotometer readings are shown in Table II.

On the basis of these data it was decided that, if any inhibiting factor or proteolytic factor was present in the tissue, it must be present in low concentration. It was decided, therefore, to reduce the concentrations of the reagents by a factor of five. In this way the relative concentration of the tissue homogenate could be increased while using a biopsy specimen within reasonable size. The tissue:saline ratio for the preparation of the homogenate was set at 37 mgm. of tissue: 2 ml. of saline. Table III illustrates the protocol by which the protease activity of the tissue specimens was measured. The results of the Biuret reaction were again recorded as % transmission by means of a Coleman Junior spectrophotometer using a 605 λ filter. These readings were then transcribed to optical density to facilitate statistical analysis.

Since pathological specimens were obtained from patients undergoing

TABLE II

TISSUE HOMOGENATE DETERMINATION

TABLE III

EXPERIMENTAL PROTOCOL

TABLE II

LET STAND 20 MIN. SHAKE					INCUBATE 38° C SHAKE	MIX LET STAND CENTRIFUGE			SHAKE INCUBATE CENTRIFUGE DECANT		% TRANSMISSION	
Tube	Homo-genate	Saline 1	Saline 2	Working Enzyme	Casein	TCA 10%	Digest		NaOH	$CuSO_4$	Trial 1	Trial 2
1	5	0	2.5	2.5	15	3	5		1	1	26.5	31.0
2	4	1	3.5	2.5	15	3	5	PIPETTE	1	1	28.2	38.0
3	3	2	4.5	2.5	15	3	5	5 Ml.	1	1	28.2	32.0
4	2	3	5.5	2.5	15	3	5	SUPER-	1	1	28.8	32.5
5	0	5	7.5	2.5	15	3	5	NATANT	1	1	28.0	32.5
6	0	7.5	10.0	0	15	3	5		1	1		84.0

TABLE III

LET STAND 20 MIN. SHAKE				INCUBATE 38° C SHAKE	MIX LET STAND CENTRIFUGE			SHAKE INCUBATE CENTRIFUGE DECANT		% TRANSMISSION
Tube	Homo-genate	Saline	Working Enzyme	Casein	TCA 10%	Digest		NaOH	$CuSO_4$	
Specimen	1	0.5	0.5	3.0	3	5	PIPETTE	1	1	
Control	0	1.5	0.5	3.0	3	5	5 Ml.	1	1	
Blank	0	2.0	0	3.0	3	5	SUPER-NATANT	1	1	

periodontal treatment, it was possible to obtain larger tissue specimens. Therefore, it was decided to prepare two samples from each pathologic specimen. One was set at the concentration designated in the preceding paragraph and the other was made at approximately two times as great a concentration. Thus the tissue:saline ratio of the homogenate was about 75 mgm. of tissue:2 ml. of saline. Results were recorded as mentioned above.

Following preliminary examination of these results, additional normal specimens were treated in the same manner as described for the pathological specimens. In order to make it possible to obtain sufficient tissue for two samples of each normal specimen, the reagent concentrations were reduced by a factor of two. Thus the necessary tissue homogenate concentrations were made possible with a biopsy specimen of reasonable size. Since the amount of the final solutions was reduced, it was necessary to use a microcuvette with the spectrophotometer to record the results.

Because two different size cuvettes were ultimately used in this study, it was necessary to establish a cuvette factor. This was accomplished by subjecting the established enzyme:substrate model to the procedure described. The quantity of protein hydrolysis products produced were measured in both types of cuvettes and recorded as % transmission. These results were converted to optical density. From these data a cuvette factor of 1.99 was calculated. This then enabled this investigator to correlate the spectrophotometric readings obtained with the microcuvette with those obtained with the larger cuvettes used in the major portion of this study.

CHAPTER IV

FINDINGS AND STATISTICAL ANALYSIS

The results of the experimental procedure described above have been separated into five groups. The spectrophotometric readings were converted into units of activity by multiplying each reading by 100. The data were statistically analyzed. The data of group I are shown in Table IV and the data of groups II, III, IV and V are shown in Table V.

Group I consists of the results of the hydrolysis of the control enzyme : substrate models. As can be seen from Table III, a control model was included each time that an experimental specimen was analyzed. The summation of these individual control models provided the control group for the experiment. A total of sixteen control models were used in the experiment and the resulting figures were grouped in a range from 23.66 activity units to 56.07 activity units. Upon examination of these figures in chronological order as can be seen from Table IV, it was evident that after subject #5 a sharp reduction in the amount of hydrolytic activity occurred. This has been attributed by this investigator to a reduction in the activity of the enzyme which was used in this study. A period of about two months had elapsed from the beginning of experimentation to that point. After that time the activity of the enzyme was more constant. Since the change in enzyme activity would have the same effect as an alteration in enzyme concentration in the control models, a correction factor was determined. The control models of subjects #1 to #5 were averaged;

TABLE IV

CHRONOLOGICAL ORDER OF EXPERIMENTAL DATA

TABLE IV

SUBJECT	WEIGHT mg.	CLINICAL EVALUATION	HISTOLOGIC EVALUATION	UNITS OF ACTIVITY		
				CONTROL	BLANK	HOMOGENATE
#1	36.9	normal	normal	50.86	6.70	a)50.72 b)45.84 (1)
#2	70.7	normal	normal	" (2)	"	a)48.81 b)44.37
#3	33.2	normal	normal	56.07 49.49 (3)	8.62 9.69	53.31
#4	29.3	normal	normal	"	"	53.46
#5	30.9	normal	normal	"	"	53.76
#6	27.6	normal	normal	32.79 31.43	4.58	32.79
#7	69.4	periodontitis	chronic inflammation	33.72	7.57	a)31.88 b)33.72
#8	49.7	"	"	"	"	a)33.72 b)35.65
#9	26.8	"	"	23.66 32.79	7.47 7.06	42.02
#10	73.6	"	"	29.16	8.36	a)30.10 b)27.57
#11	43.3	"	"	28.40	8.62	35.65
#12	30.2	"	"	37.68 32.79	7.57 6.05	37.68
#13 (4)	21.1	normal	normal	26.61 27.80 25.97 25.97	7.20 9.11	27.80
#14	23.7	normal	normal	"	"	28.40
#15	22.8	normal	normal	"	"	29.59

(1) This indicates that two samples of homogenate solution were treated individually and the readings recorded.

(2) This indicates that the preceding control and blank were used in the analysis of data of two or more specimens analyzed on the same day.

(3) This indicates that more than one control and blank were analyzed with a particular group of subjects.

(4) This subject and following subjects were analyzed by a microcuvette method (and the data is corrected for the determined cuvette factor.

TABLE V

GROUPED EXPERIMENTAL DATA

TABLE V

GROUP II NORMAL SPECIMENS			
SUBJECT	WEIGHT	UNITS OF ACTIVITY	
		CONTROL	HOMOGENATE
#1	36.9	26.76*	26.70* 24.13*
#2	70.7	"	23.35* 25.69*
#3	33.2	29.51* 26.05*	28.06*
#4	29.3	"	28.14*
#5	30.9	"	28.30*
#6	27.6	32.79 31.43	32.79
#13	21.1	26.61 27.80 25.97	27.80
#14	23.7	25.97 "	28.40
#15	22.8	"	29.59

*corrected for decrease in enzyme activity

GROUP III PATHOLOGIC SPECIMENS			
SUBJECT	WEIGHT	UNITS OF ACTIVITY	
		CONTROL	HOMOGENATE
#7	69.4	33.72	31.88 33.72
#8	49.7	"	33.72 35.65
#9	26.8	23.66 32.79	42.02
#10	73.6	29.16	30.10 27.57
#11	43.3	28.40	35.65
#12	30.2	37.68 32.79	37.68

GROUP IV NORMAL SPECIMENS			
SUBJECT	WEIGHT**	UNITS OF ACTIVITY	
		CONTROL	HOMOGENATE
#13	30.0	26.61 27.80 25.97	20.38
#14	36.2	25.97 "	20.38
#15	32.7	"	21.47

GROUP V PATHOLOGIC SPECIMENS			
SUBJECT	WEIGHT**	UNITS OF ACTIVITY	
		CONTROL	HOMOGENATE
#9	41.4	23.66 32.79	28.40
#10	137.4	29.16	13.67
#11	77.8	28.40	24.36
#12	46.8	37.68 32.79	18.71

** The concentration of the homogenate solution was increased by a factor of two by adjustment of the tissue weight and the saline solution.

the control models of subjects #6 to #15 were averaged. The controls which were obtained during the period of greater enzyme activity were reduced by a factor of 1.9 which was obtained by dividing these averages. The mean for the entire group of control models after this correction was 29.44 units of activity. The median was 28.78 units of activity. The standard deviation for a small sample (S_x) was found to be 4.27 units of activity. The standard error of the mean (S.E.$_{\bar{x}}$) for the control group was 1.07 units of activity.

Specimens from normal gingiva which were analyzed at the homogenate concentration of 37 mg. of tissue to 2 ml. of saline were included in Group II. A total of nine specimens varied in a range from 24.52 activity units to 32.79 activity units with a mean activity of 28.11 activity units. When each specimen was compared to its related control model, little difference could be noted between the two. Seven of the group showed a slight intrinsic proteolytic activity. This was indicated by the fact that the number of activity units was higher for the specimen than for the control. Two specimens exhibited a slight inhibitory action in respect to their individual controls as evidenced by lower activity readings. Group II normal specimens had a median of 28.14 activity units. The standard deviation (S_x) was 2.58 units. The standard error of the mean (S.E.$_{\bar{x}}$) was 0.79 units. The slight inhibitory activity, which is indicated by a comparison of the means of group I and group II, is not statistically significant. This was determined by the application of the t test to these data. The results of the test being $t_{23} = 1.000$ which,

when applied to probability can be expressed as $0.4 < P < 0.3$. In this instance, a difference as large or greater would occur by chance between three and four times out of ten. The clinical evaluation of normal gingiva was substantiated by histological section in each case.

Seven gingival specimens which clinically and histologically exhibited a chronic inflammatory condition and whose tissue homogenate concentration was 37 mg. of tissue to 2 ml. saline were placed into group III. Of these specimens one was eliminated from the final analysis leaving a total of six specimens. The single specimen was discarded because of the extreme behavior of the tissue homogenate upon the enzyme:substrate mixture and of the respective control models. Like the normal specimens discussed above each pathologic specimen was closely related to its respective control. Four of the specimens showed an intrinsic proteolytic activity and two of the specimens had an inhibitory effect. However, dissimilarities were manifest when the group was considered in its entirety. Group III had a mean of 35.28 units of activity and a median of 35.17 units of activity. The standard deviation was 5.26 units. The standard error of the mean ($S.E._{\bar{x}}$) was 1.91 units of activity. The variance between group III and group I has been shown to be statistically significant by means of the t test. Results of this test show $t_{20} = 2.666$. In respect to probability this can be expressed as $0.02 < P < 0.01$. Therefore, the probability that a difference as great or greater would occur by chance is between one and two times in a hundred.

When group II and group III were compared, they were also found to

differ significantly. When the t test was applied, a result of $t_{13} = 3.463$ was obtained. The probability was shown to be $0.01 < P < 0.001$. A difference of this magnitude then would occur by chance only between one in one hundred times and one in one thousand times.

Group IV was comprised of three specimens which were taken from subjects who were included in group II. These specimens for group IV were actually a part of the original biopsy sample from a subject in group II. The gingival tissue was histologically and clinically normal. The tissue homogenate concentration, however, was increased two-fold. Each of the three samples treated in this manner exhibited a decrease in enzymatic activity in respect not only to its control model but especially to its "mate" in group II. This was signified by a decrease in the number of activity units observed. The mean of this group was 20.74 units of activity. The median was 20.38 units. The standard deviation (S_x) was 0.88 units of activity and the standard error of the mean ($S.E._{\bar{x}}$) was 0.51 units of activity. Although group IV was of a very small sample, the probability that groups II and IV were of separate universes, was statistically greater than 0.001. Only in one in one thousand times would this magnitude of difference occur by chance.

Each of the four specimens in group V was also taken from a particular subject already included in group III. The specimens were of chronically inflamed gingiva. The only variation again was a two-fold increase in the tissue homogenate concentration. The mean of 21.19 units of activity differed greatly from the mean of group III which was 35.28 units of activity. The standard deviation was 0.72 units and the standard error

of the mean was 0.51 units of activity. The variation between these two groups was statistically significant as illustrated by the results of the t test. For a value of the relative deviate (s/x) equal to 3.522 t_8 was shown to be $0.01 < P < 0.001$. Therefore, a difference as great or greater would occur by chance between one in one hundred and one in one thousand times.

Groups IV and V were also analyzed statistically for their relationship to each other. Differences between them were found not to be significant. The value of the relative deviate was 0.1264. When this is applied to the t test for five degrees of freedom, the probability of this occurring by chance is greater than nine times out of ten.

CHAPTER V

DISCUSSION

Turning for a moment to the first chapter of this report, it will be remembered that an important aspect of this study was the development of a method of investigation. The protocol described in Chapter III illustrates a method which has proven to be feasible for measuring proteolytic enzyme activity or proteolytic enzyme inhibitor activity in gingival tissues. The usefulness of the method to measure proteolytic activity was shown by the results of group III. The ability to detect proteolytic enzyme inhibition was evident in groups IV and V. The results of the control group, group I, were obtained through the application of methods described in the literature and by means of studies of hydrolysis of casein by chymotrypsin. The latter were performed by accepted procedures. Significant differences were obtained between group I and groups III, IV, and V in which the tissue homogenates were the only variants.

Once again, let us classify the five groups of data. Group I consists of the results of the hydrolysis of the control enzyme: substrate models. The constituents of the control models are shown in Table III. The results of the effect of tissue homogenates of normal gingival specimens upon an enzyme : substrate model were included in Group II. The homogenate concentration of this group was set at 37 mg. of tissue to 2 ml. of saline. Group III was identical to Group II except that the effects of chronically inflamed gingivae were measured instead of normal gingivae. In Group IV the effects of normal gingival homogenates upon the enzyme:

substrate model were measured. The gingival specimens were obtained from subjects already included in Group II. However, the concentration of the homogenate solution was now increased by a factor of approximately two by increasing the weight of the specimen and adjusting the amount of saline in the mixture. The results of Group V were obtained from chronically inflamed specimens of subjects included in Group III. The homogenate concentration of the specimens was increased in the same manner as in Group IV.

Although five distinct groups were delineated in Chapter IV, it will be necessary to correlate these groups to extract the salient ideas derived from this investigation. Initially, it would seem that the normal tissue specimens in group II had no effect upon the enzyme chymotrypsin during the period of incubation. The spectrophotometric readings of group III indicated a greater amount of products of hydrolysis in the homogenate enzyme substrate mixture as compared to the control group. Since these differences were statistically significant, an intrinsic proteolytic enzyme with an action upon the substrate, casein, was present in the inflammatory tissue specimens of group III. No evidence of a possible enzyme and enzyme inhibitor relationship can be shown until the results of groups IV and V are simultaneously considered.

Group IV samples demonstrated a significant inhibitory effect upon the enzyme chymotrypsin when they were compared to their "mates" in group II. This was true not only when the groups were considered as a whole and analyzed statistically but also when the "mated" samples were con-

sidered individually between groups. These results can be seen by comparing these groups in Table V. This same phenomenon was evident between groups III and V as shown in Table V.

To what can these differences be attributed? The presence of an enzymatic inhibitory factor is definitely evident. The enzyme inhibition can be detected after a change in concentration of the tissue samples. This may indicate that at the lower concentrations, the enzyme inhibitor effect of the tissue was not measurable by this method. However, by increasing the concentration of the tissue homogenate the presence of an inhibitor could be demonstrated in both normal and inflamed tissue. Yet, this conclusion alone does not provide an adequate reason for the significant differences between groups III and V. Group III demonstrated an intrinsic proteolytic activity. These data were significantly different from group II as well as the control group, group I. When the concentration of the tissue homogenates was increased as in groups IV and V, the inhibitor activity of both groups showed no significant differences. In fact, the differences in the levels of proteolytic enzyme activity of normal and inflamed tissues were no longer apparent. This would seem to indicate a higher degree of inhibitor activity in inflamed tissues than in normal tissues.

On the other hand, the study may be understood more clearly by examining it from another aspect. The data from groups IV and V indicate the presence of an inhibitor in both normal and chronically inflamed gingival tissues. The fact that the normal and inflamed tissues of groups

II and III, which were analyzed at a lower concentration, did not show the presence of an enzyme inhibitor can be attributed to a balanced relationship of enzyme to inhibitor. This relationship between enzyme and inhibitor might not be numerically equivalent. That is, it might require more inhibitor to counteract a given amount of enzyme. In fact, this seems to be true when the normal and pathological groups of lower tissue concentrations are considered in relation to the normal and pathologic groups of higher tissue concentration. If, for instance, a relationship of one part enzyme to two or more parts enzyme inhibitor produced the results of group II or III, then, by increasing the concentration of the tissue homogenate of the same subject, the net result would be one of absolute enzyme inhibition. This is illustrated in the diagrams in figures 3 and 4.

An *in vivo* interpretation of an enzyme : inhibitor mechanism of this nature is depicted in figure 3. Figure 4 depicts an analysis of the *in vitro* situation as applied to this experiment. It can be seen that by keeping the amount of the experimental enzyme, chymotrypsin, constant while varying the concentration of the tissue components, the absolute inhibitor activity is altered. This alteration under experimental manipulation would be depicted as an appearance of inhibitor activity. This inhibitor activity would be approximated to the difference between the absolute inhibitor activity *in vivo* and the absolute inhibitor activity *in vitro*.

Therefore, analyzing the problem in this manner, the results of groups II and IV become clearer. That is, in normal gingival tissue a

FIGURE III

ENZYME : INHIBITOR RELATIONSHIP

IN VIVO

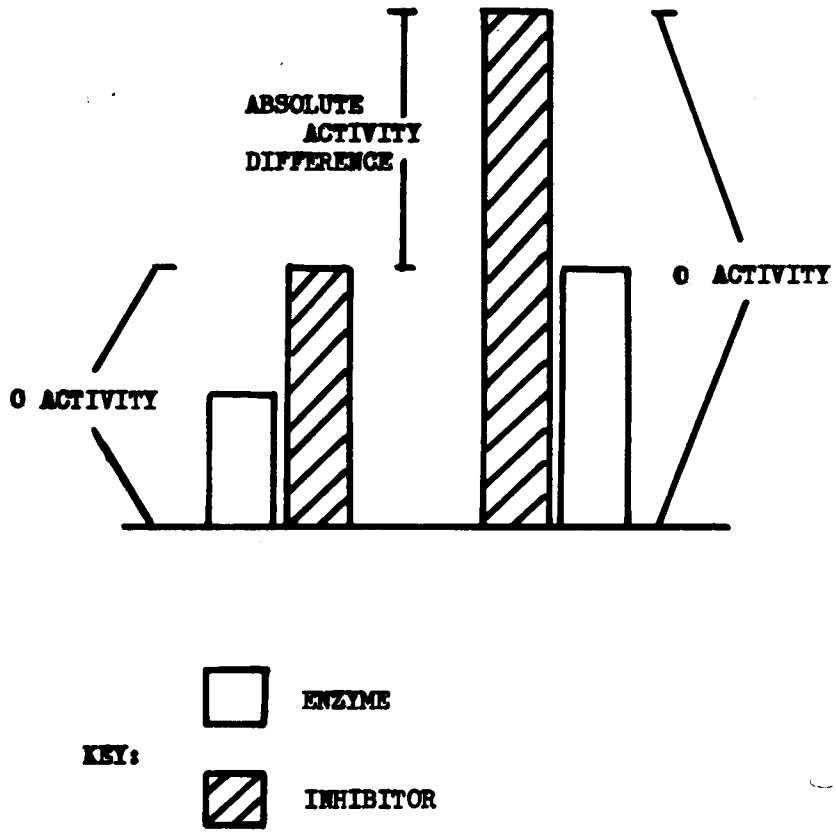

FIGURE 3

ENZYME : INHIBITOR RELATIONSHIP IN VIVO

FIGURE IV

ENZYME : INHIBITOR RELATIONSHIP

IN VITRO

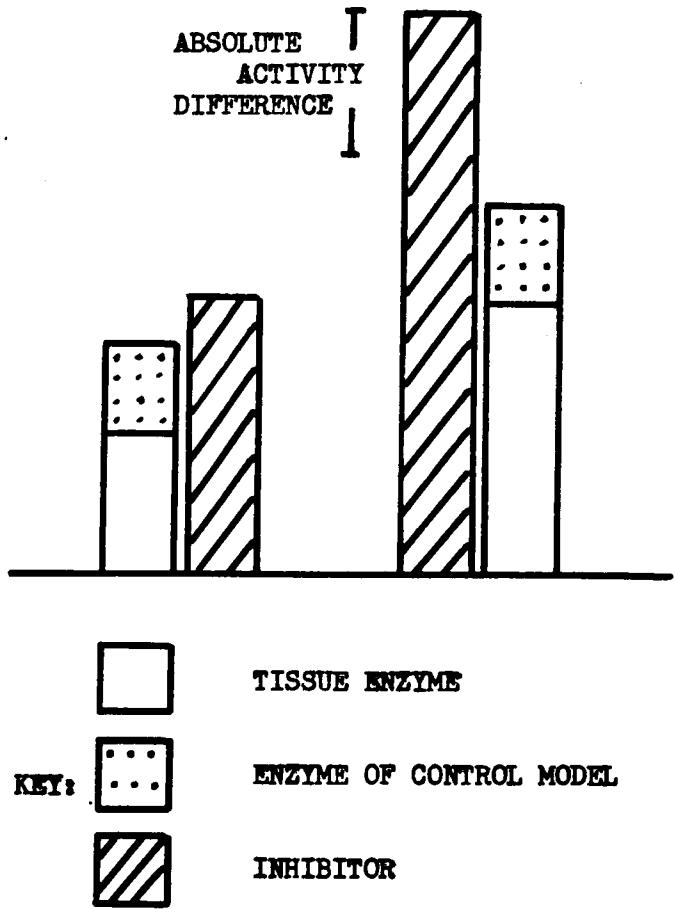

FIGURE 4

ENZYME : INHIBITOR RELATIONSHIP IN VITRO

balanced enzyme-antienzyme mechanism would show no effect upon the enzyme substrate mixture. Yet, the same tissue specimen at greater homogenate concentration would produce an inhibitory effect upon a similar enzyme substrate mixture. The results of groups III and V seem to indicate that an enzyme - inhibitor relationship would be a dynamic fluctuating system just as it is true of many of the bodily processes. In general the tissues in group III were found to exert an additive proteolytic effect upon the enzyme substrate mixture when compared to group I. Yet, upon increasing the tissue homogenate concentration, an inhibition was evident in amounts great enough so that no significant differences between groups IV and V could be found. It will be remembered that the "mates" of these groups, groups II and III differed significantly. Those findings are attributed to an overcompensation of the inhibitory factors in response to the proteolytic enzymes produced in the inflammatory process.

These ideas have basis in the literature. West and Hilliard (1949) indicated that serum proteolytic inhibitor levels remained elevated during certain disease states and when the tissues were undergoing an adjustment to a normal state. Tokuda (1959) showed that monocytes subjected to an antigen-antibody reaction secreted a protease and then a protease inhibitor. That latter work would seem to indicate the presence of a time relationship in that mechanism not only between the proteases and the inhibitors that were produced but also between cells or groups of cells which were subjected to injury. In this way neither enzyme nor

inhibitor would act alone. Therefore, it is understandable that in a proteolytic enzyme and inhibitor mechanism of this type, a relationship such as the one described in this present study could produce the results that were obtained.

From this investigation questions arise which must be answered by further research. The nature of the inhibitor must be determined. The degree to which the inhibitory factor measured in tissues is influenced by the proteolytic enzyme inhibitors in serum should be elucidated. Are results such as those obtained in groups II and IV of this study attributable to the fact that even so-called normal gingival tissues exhibit a chronic inflammation to varying degrees? Only through a continuing quest for new ideas can knowledge in any field progress.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the activity of proteolytic enzymes in clinically and histologically normal gingival tissues as well as chronically inflamed gingivae. The experimental procedures were designed to demonstrate any possible relationship of proteolysis to proteolytic enzyme inhibition. Due to a minimum of information in the literature regarding a specific method of investigation, the development of such a method was considered important.

Chapter III describes a method of investigation by which proteolytic enzyme activity or proteolytic enzyme inhibition of a gingival tissue specimen was measured. The procedure required homogenation of the tissue and incubation of the homogenate with a known amount of a proteolytic enzyme. Chymotrypsin was used in this study. The mixture of enzyme and homogenate was then incubated with a known quantity of substrate for the enzyme. The products of hydrolysis were then measured colorimetrically after employing the Biuret reaction. Enzyme : substrate control models were employed as a basis for determining the proteolytic or inhibitory activity of the tissue specimens. The method was workable using relatively small tissue samples.

Nine normal gingival specimens and six chronically inflamed gingival specimens from fifteen subjects were analyzed using the method described. Significant differences were demonstrated between the

effects of normal and inflamed gingivae upon the enzyme : substrate mixture. This was attributed to the proteolytic activity of the inflammatory process. The presence of an inhibitor of chymotrypsin was demonstrated in both normal and chronically inflamed human gingivae. This was accomplished by varying the concentration of the homogenate of tissue specimens from individual subjects. The relationship between an enzyme and its inhibitor, which could produce the results that are reported in Chapter IV, was discussed.

The following conclusions seem to be justified on the basis of the results of this study:

1) The method of investigation which was developed is applicable to this study.

2) The presence of a proteolytic enzyme inhibitor was demonstrated both in normal and inflamed gingivae.

3) The tissues from inflamed gingiva seem to have a higher proteolytic enzyme inhibitor level than do corresponding samples from normal gingiva. This conclusion is based on the observation that the additional proteolytic component ascribed to the homogenate sample is minimized or eliminated by elevating the tissue homogenate level in the experimental procedure. Such a reversal in proteolytic behavior was not demonstrable for the normal tissue homogenates.

BIBLIOGRAPHY

I. PRIMARY SOURCES

Aisawa, N. Studies on the Protease Inhibitor in Human Blood. II. Zone Electrophoresis Studies on Protease Inhibitor in Human Blood, Yokohama Medical Bulletin 11: 206-211, 1960.

Astrup, Tage, and Stage, Agnete. A Protease Inhibitor in Ox Lung Tissue, Acta Chemica Scandinavica, 10: 617-622, 1956.

Bodman, John. Measurement Physiological Importance and Control of Proteolytic Enzyme Inhibitors, Clinica Chimica Acta 3: 108-110, 1958.

------, The Measurement of Serum Inhibitor to Chymotrypsin, Its Nature and Clinical Significance, Rheumatism 13: 25-44, 1957.

Camus, L., and Gley, E. Action Du Serum Sanguin Sur Quelques Ferments Digestifs, Comptes Rendus Des Seances Et Memoirs de La Societe De Biologie 49: 825, 1897.

Dillard, George, and Chanutin, Alfred. The Protease and Antiprotease of Plasmas of Patients with Cancer and Other Diseases, Cancer Research 9: 665-668, 1949.

Dutherie, E.S., and Lorenz, L. Protease Inhibitors I. Assay and Nature of Serum Antiprotease, Biochemistry Journal 44: 167-173, 1949.

Grob, David. The Antiproteolytic Activity of Serum I. The Nature and Experimental Variation of the Antiproteolytic Activity of Serum, Journal of General Physiology 26: 405-421, 1942-43.

-----, Proteolytic Enzymes I. The Control of Their Activity, Journal of General Physiology 29: 219-247, 1945-46.

Hildebrandt, H. Weiteres uber hyd rolytische Fermente' deren Schicksal und Wirkungen, Sowie uber Fermentfestigkeit und Hemmung der Dermentationen im Organismus, Virchows Archiv fur Pathologische Anatomie und Physiologie und klinische Medizin, 131: 5, 1893.

Hort, E.C. Diagnosis of Cancer by Examination of Blood, British Medical Journal 2: 966-971, 1909.

Hussey, R.G., and Northrop, J.H. A Study of the Equilibrium Between the So-called Antitrypsin of the Blood and Trypsin, Journal of General Physiology 5: 335-351, 1922-23.

Kunitz, M., and Northrop, John H. Isolation From Beef Pancreas of Crystalline Trypsinogen, Trypsin, a Trypsin Inhibitor and an Inhibitor-Trypsin Compound, Journal of General Physiology 19: 991-1007, 1935-36.

Landsteiner, Karl. Zur Kenntnis der antifermentativen lytischen und agglutinierenden Wirkungen das Blutserums und der Lymphe, Centralblatt F. Bakteriologie Parasitenk u. Infektionskrank heiten 27: 357, 1900.

Opie, Eugene L. Enzymes and Antienzymes of Inflammatory Exudates, Journal of Experimental Medicine 7: 316-334, 1905.

Rush, Benjamin, Jr., and Cliffton, Eugene E. Control of Proteolytic Activity in Serum; Effect of Soybean Inhibitor In Vivo in the Mouse, American Journal of Physiology 166: 485-491, 1951.

Scevola, M.E., Novati, C.C., Felisati, D. Distibuzione Degli Inibitori Verso La Tripsine E La chimotripsina In Tessuti Di Differenti Specie Animali, Bollettino Della Societa Italiana Di Biologica Sperimentale 30: 261-263, 1954.

Scevola, M.E., and Felisati, D. Distribution of Trypsin and Chymotrypsin Inhibitors in Normal and Tumoral Tissues of Different Species, Biochimica Applicata 2: 83-94 1955.

Tauber, Henry. Chymotrypsin Inhibition by Human Serum in Health and Disease, Proceedings of the Society for Experimental Medicine and Biology 74: 486-489, 1950.

Tokuda, Akira Biochemical Studies on Cellular Antigen Antibody Reaction in Tissue Culture. II. Release of Protease Inhibitor, Mei Medical Journal 9: 217-225, 1959.

Ungar, Georges and Adler, Barr. A Serum Peptidase and Its Possible Role in Protease Inhibition, Proceedings of the Society for Experimental Biology and Medicine 91: 231-234, 1956.

Veremeenko, K.M. Inhibition of Trypsin Activity by Blood Plasma, Ukrains'kii Biokhemichnii Zhurnal 32: 793-805, 1960.

West, P.M., and Hilliard, J. Proteolytic Enzyme Inhibitors of Human Serum in Health and Disease, Proceedings of the Society for Experimental Biology and Medicine 71: 169-172, 1949.

II. SECONDARY REFERENCES

Aizawa, N. Studies on the Protease Inhibitor in Human Blood I. Assay Method and Some Chemical Properties, Yokohama Medical Bulletin 11: 101-110, 1960.

Albrechtsen, O.K., Storm, O., Claassen, M. Fibrinolytic Activity of Some Human Body Fluids, Scandinavian Journal of Clinical and Laboratory Investigation 10: 310-318, 1958.

Astrup, Tage and Albrechtsen, O.K. Estimation of the Plasminogen Activator and the Trypsin Inhibitor in Animal and Human Tissues, Scandinavian Journal of Clinical and Laboratory Investigation 9: 233-243, 1957.

Austen, K.F., Brocklehurst, W.E. Inhibition of the Anaphylactic Release of Histamine from Chopped Guinea Pig Lung by Chymotrypsin Substrates and Inhibitors, Nature (London) 186: No. 4728 1960.

Benditt, Earl, P., Arase, Margaret, Enzyme in Mast Cell with Properties Like Chymotrypsin, Journal of Experimental Medicine 110: 451-460, 1959.

Bull, Henry B. Physical Biochemistry John Wiley & Sons, Inc. New York, 1943.

Glenner, G.G., Cohen, L.A. Histochemical Demonstration of a Species Specific Trypsin-like Enzyme in Mast Cells, Nature (London) 185: 846-847, 1960.

Grob, David. Proteolytic Enzymes III. Further Studies on Protein, Polypeptide and Other Inhibitors of Serum Proteinase, Leucoproteinase, Trypsin and Papain, Journal of General Physiology 33: 103-124, 1949-50.

Herberts, G. Serum Inhibiting Factors in Anaphylactic Shock, Otolaryngology Suppl. 140: 145-151.

Jacques, R. and Schar, B. Action of Enzymes on Isolated Smooth Muscle Organs and Cells with Special Attention to Proteolytic Enzymes, Helvetica Physiologica Et Pharmalogica Acta 15: 134-149, 1957.

Jasmin, G. and Robert, A. The Mechanism of Inflammation : An International Symposium. Acta, Inc. Montreal, 1953.

Mehler, Alan H. Introduction to Enzymology. Academic Press Inc. New York, 1957.

Menkin, Valy. Biochemical Mechanisms in Inflammation. Charles C. Thomas, Springfield, Illinois, 1956.

Northrop, John H., Kunitz, M. Crystalline Trypsin III. Experimental Procedures and Methods of Measuring Activity, Journal of General Physiology 16: 313, 1932-33.

Sale, E.E., Priest, S.G., Jensen, H. Studies on the Antiproteolytic Activity of Bovine Blood, Journal of Biological Chemistry, 227: 83, 1957.

Schwartz, Bernard, Schwartz, Joyce. A Review of the Biochemistry and Pharmacology of Alpha-Chymotrypsin, American Academy of Opthalmology and Otolaryngology 64: 17-24, 1960. Supplement.

Shulman, N. Raphael. Studies on the Inhibition of Proteolytic Enzymes by Serums. II. Demonstration That Separate Proteolytic Inhibitors Exist in Serum, Their Distinctive Properties and Specificity of Their Action, Journal of Experimental Medicine 95: 593-603, 1952.

-----, Studies on the Inhibition of Proteolytic Enzymes by Serums I. The Mechanism of the Inhibition of Trypsin Plasmin and Chymotrypsin by Serum Using Fibrin Tagged with I^{131} as a Substrate, Journal of Experimental Medicine 95: 571, 1952.

Sizer, Irwin W. Chemical Aspects of Enzyme Inhibition, Science 125: 54-59, 1957.

Tokuda, Akira Biochemical Studies on Cellular Antigen-Antibody Reaction in Tissue Culture. I. Release of Certain Protease, Mei Medical Journal 9: 201-215, 1959.

Ungar, Georges Inflammation and Its Control - A Biochemical Approach, The Lancet 2: 742-746, 1952.

Wells, George C., Babcock, Carvelle Epidermal Protease, Journal of Investigative Dermatology 21: 459-463, 1953.

Zweifach, B.W., Nagler, A.L., Troll, W. Some Effects of Proteolytic Inhibitors on Tissue Injury and Systematic Anaphylaxis, The Journal of Experimental Medicine 113: 437-450, 1960.