June 1984

Collagenolytic Activity in Oral Microorganisms

Marilyn Susan Lantz

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COLLAGENOLYTIC ACTIVITY IN ORAL MICROORGANISMS

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A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Dental Science
at
The University of Connecticut
1984
Acknowledgements

I would like to express my gratitude to the members of my thesis committee:

To Dr. Paul Robertson for his kindness, guidance, support and friendship, and especially for allowing me the freedom to pursue areas of interest that led me off the "beaten path";

To Dr. Kenneth Kornman for introducing me to the world of anaerobes, for his guidance in the microbiological aspects of this research and especially for his friendship; and,

To Dr. Barbara Kream for her critical reading of this manuscript and for her many valuable suggestions for its improvement.

I would like to thank Dr. Bruce Rutherford for serving as outside reader and for his helpful suggestions regarding the treatment of data presented in this manuscript.

The completion of this work would have been impossible without the expert technical assistance of Michele Menard. Her outstanding abilities, patience and dedication are much appreciated.

Much secretarial help was required in the preparation of this manuscript and I am indebted to Beverly Kranmas and Darlene Jeanetti for their assistance.

I wish to thank Dr. Magnus Höök and Dr. Lech Switalski for sharing their scientific expertise and inviting me to work with them at the University of Alabama in Birmingham. Their assistance in performing the laminin binding studies was invaluable.
Finally, I want to thank my daughter Maya, for being my closest friend, for offering inspiration and for her ever-ready smile.
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Introduction

The predominant forms of periodontal disease, gingivitis and periodontitis, affect almost all persons with natural teeth, and on a world-wide basis, periodontitis constitutes the leading cause of tooth loss (Ramfjord et al. 1968; Johnson, Kelly and VanKirk 1962). A great body of epidemiological and experimental evidence has established that accumulation of microorganisms adjacent to the gingiva leads to the initiation and maintenance of gingivitis in humans (Waerhaug 1971; Loe, Theilade and Jensen 1965; Theilade et al. 1966; Loe 1971) and it is well established that dental plaque, a tooth adherent material composed of microorganisms, is the main etiological agent in gingivitis and periodontitis. Recent reviews (Robertson 1983, Page and Schroeder 1982) have suggested that a number of periodontitis subclasses exist. These include chronic adult periodontitis, aggressive (rapidly progressing) periodontitis and juvenile periodontitis. All forms of periodontitis result in alveolar bone resorption, loss of periodontal ligament attachment to cementum and pocket formation. In addition, suppuration and bleeding may occur. The consequences of loss of tooth support are increasing mobility and eventual exfoliation of the tooth. In chronic adult periodontitis, three events occur over a long period of time and appear to effect most of the dentition of the individual. Loss of support rarely exceeds 0.3 mm. per year and is usually associated with plaque and calculus (Robertson 1983). Aggressive adult periodontitis is associated with rapid loss of periodontal
support in young adults and the severity of periodontal destruction observed is inconsistent with the minimal plaque and calculus usually observed. Juvenile periodontitis, which occurs typically in adolescents, is characterized by rapid loss of periodontal support usually confined to permanent molars and incisors, although more generalized patterns have been observed. Juvenile periodontitis patients also typically exhibit minimal plaque and calculus (Robertson 1983).

**Microbial Etiology of Periodontal Disease**

While clear patterns of pathogenic oral organisms for each of the periodontal diseases have not been firmly established, the available evidence favors the role of indigenous organisms rather than a more classical infection by particular exogenous organisms (Loesche 1976). In addition, a large body of evidence suggests that gingival health as well as the various forms of gingivitis and periodontitis are associated with plaques of different microbial composition.

It appears that healthy sites are usually found associated with plaques consisting mainly of Gram-positive cocci, predominantly *Actinomyces* and *Streptococci* (Listgarten 1976; Slots 1976; Slots 1977; Listgarten and Hellden 1978; Moore et al. 1982c) whereas plaques associated with non-bleeding gingivitis appear to contain relative increases in the percent *Actinomyces* species at the expense of *Streptococci* (Loesche and Syed 1978). One recent study has implicated *Actinomyces naeslundii*. 
Actinomyces odontolyticus, Fusobacterium nucleatum, Lactobacillus D-2, Streptococcus anginosus, Veillonella parvula and Treponema species A, as the most likely etiologic agents of experimental gingivitis in humans (Moore et al. 1982a), while plaques associated with gingivitis in pregnancy ("bleeding gingivitis") appear to have increased levels of Bacteroides melaninogenicus ss. intermedius (Kornman and Loesche 1980; Jensen et al. 1981). Plaques associated with chronic adult periodontitis appear to have increased levels of Bacteroides gingivalis (Slots 1977; Spiegel et al. 1979; White and Mayrand 1981; Zambon et al. 1981; Kornman et al. 1981; Slots 1982) whereas Actinobacillus actinomycetemcomitans and Capnocytophaga appear to be associated with juvenile periodontitis (Slots 1976; Tanner et al. 1979; Slots et al. 1980; Slots 1982). Bacteroides capillus (Kornman and Holt 1981), which may be identical to Bacteroides buccae (Holdeman et al. 1982), may be associated with localized severe periodontitis in adults. One recent study has cited Fusobacterium nucleatum and Eubacterium timidum and nodatum to have the highest incidence among species most frequently cultivated from affected sulci of young adult severe periodontitis patients (Moore et al. 1982b).

Thus, it appears that, in general, the change from health to disease involves a shift in plaque microflora from one consisting predominantly of Gram-positive cocci to one consisting predominantly of Gram-negative anaerobic and facultative bacteria. In addition, there is substantial evidence suggesting an etiological
role for *Bacteroides* in some forms of gingival and periodontal pathosis.

**Collagen Degradation in Periodontal Disease**

A major feature of periodontal disease from initial to advanced stages, is a marked reduction in gingival collagen fiber density (Page 1972; Page and Schroeder 1973). Collagen, a structural protein comprising 30% of the body protein in mammals, is a long, rod-like molecule consisting of three parallel polypeptide chains wound about each other in a left handed poly-L-proline type of helix (Traub and Piez 1971). A characteristic of the collagen polypeptide chain (alpha-chain) is that the amino acid, glycine, occurs at every third residue of the main body chain and in addition, the amino acids proline and hydroxyproline predominate (Gallop et al. 1972). Each triple helix (gamma-chain) is twisted in a rope-like fashion (termed a coiled coil) and has a molecular weight of approximately 300,000 (Harper 1980). The molecule is stabilized both by hydrogen bonding and intra- and intermolecular crosslinks (Tanzer 1973). There are several different types of collagen (Bornstein and Sage 1980). Type I collagen, the most common form, is found in a large variety of tissues and predominates in skin. It has the structure \( \alpha_1(I) \times \alpha_2(I) \). A Type I trimer \( \alpha_1(I) \times \alpha_2(I) \) has also been observed in skin. Relative increases in this type of collagen have been reported to occur in subgingival connective tissue of periodontitis lesions (Page and Schroeder 1973). Type II collagen, \( \alpha_1(II) \times \alpha_2(II) \), is found predominately in cartilage and Type III
collagen, \([\alpha_1(III)]_3\), is present in blood vessels embryonic skin, lung, muscle and liver (Harper 1980). Type I, II and III collagens collectively are called interstitial collagens (Bornstein and Sage 1980). Type IV collagen, the major basement membrane collagen, structurally resembles the interstitial collagens. However, unlike the interstitial collagens, it contains pepsin-sensitive and other protease-sensitive sites in the triple helical domain. Another collagen, Type V, has recently been described and appears also to be associated with basement membranes (Sage and Bornstein 1979). Bornstein and Sage (1980) suggest that few if any of the collagen types have a unique tissue distribution and that it is likely, as detection methods become more sensitive, that a broad distribution range will be found for each of the collagens. However, quantitative differences in the preparations of the different collagen types in local anatomic sites clearly exist and are probably important for normal tissue function. Butler et al. (1975), have found both Type I and Type III collagens in bovine periodontal ligament in a ratio of approximately 4:1.

Two principle pathways of collagen loss in periodontal disease have been suggested. The first is related to an overall decrease in collagen production (Page and Schroeder 1973). The second, which is the subject of the present thesis involves degradation of existing collagen by increased levels of collagenases elaborated by periodontal host cells and/or oral microorganisms. The triple helical region of the collagen molecule
is, in general, quite resistant to nonspecific proteases (Harper 1980). Collagenases are defined as enzymes capable of causing hydrolytic scission of peptide bonds in the helical regions of undenatured collagen at physiologic pH and temperature. Reviews dealing with enzymes meeting these criteria have appeared and include animal or vertebrate collagenase (Gross 1976; Weiss 1976, Robertson and Simpson 1976; Harris and Cartwright 1977; Perez-Tamayo 1978), microbial collagenase (Seifter and Harper 1971, Harper 1980), serine proteases (Grant et al. 1980) and a number of enzymes from lower eucaryotes (Keil 1979).

Vertebrate collagenases derived from host cells common to the periodontium and microbial collagenases elaborated by members of the oral flora may play a major role in periodontal connective tissue degradation. Vertebrate collagenase was first isolated by Gross and Lapiere (1962) in studies of tadpole metamorphosis and enzymes with similar characteristics have since been identified in about twenty animal species. Most of these enzymes have pH optima from 7 to 9, molecular weights ranging from 40,000 to 65,000 daltons, absolute requirements for calcium, and are inhibited by serum α<sub>2</sub>-macroglobulin. Vertebrate collagenases cleave undenatured collagen at a single peptide bond between residues 772 and 773 in the α<sub>1</sub> chain of the Type I molecule. This single cleavage characteristic applies to Type I, II and III collagens, although some difference in relative susceptibility among collagen types has been observed. Vertebrate collagenase activity against Type IV and V collagen is less clear although it is
likely that specific collagenases exist for each collagen type (Bornstein and Sage 1980). Several lines of evidence support participation of host vertebrate collagenase in periodontal disease. With the exception of initial gingivitis, increased levels of enzyme activity have been observed in inflamed gingiva relative to healthy gingival tissue (Fullmer 1970; Robertson and Grupe 1972). Gingival explants elaborate both an active collagenase with typical 1/4 - 3/4 cleavage characteristics and a latent enzyme which is activated by a mast cell factor (Simpson and Taylor 1974), lymphocyte factors and products of microbial plaque (Robertson et al. 1974). Polymorphonuclear leukocytes, a dominant cell type in the inflammatory infiltrate associated with periodontal disease, contain a vast array of proteolytic enzymes including collagenase (Lazarus et al. 1968). Macrophages, which increase significantly in number at an early stage of periodontal disease, are activated in the presence of endotoxins and lymphokines to produce collagenase (Wahl et al. 1974). Finally, a vertebrate collagenase has been demonstrated in gingival fluid. The enzyme activity appeared to be related to pocket depth and severity of inflammation (Golub et al. 1976).

While much progress has been made in purifying and characterizing vertebrate collagenases, information on procaryotic collagenase activity is limited and interrelationships between the two collagenolytic systems in periodontal disease are essentially unexplored. As can be seen in Table I, convincing evidence in support of specific collagenase elaboration exists for
only a few procaryotes, including species of *Bacteroides*, *Bacillus*, *Clostridium* and *Vibrio*. Further confirmation of reported collagenolytic activity is required for species of *Mycobacterium* and *Pseudomonas* (Waldvogel and Swartz 1969; Brown, Bloomfield and Tam 1974; Kessler, Kennah and Brown 1977). Among the oral microflora, collagenolytic activity has been reportedly associated with *Bacteroides melaninogenicus* (Gibbons and MacDonald 1961) and conditions for demonstration of enzyme activity were later described by Hausmann, Courant and Arnold (1962). Mayrand et al. (1980) have reported cell bound collagenolytic activity associated with oral strains of *B. asaccharolyticus* (*B. gingivalis*) but were unable to demonstrate collagen degradation by *B. melaninogenicus*. Steffen and Hentges (1981) found collagenolytic activity associated with *B. melaninogenicus* subspecies *melaninogenicus* and *intermedius* and *B. asaccharolyticus* but not with *B. fragilis*, *B. thetaiotaomicron*, and a number of species of *Fusobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus* and *Streptococcus*. This laboratory has screened numerous oral isolates for collagenolytic activity and has found no activity among species of *Fusobacterium*, *Actinomyces*, *Capnocytophaga*, and *Selenomonas* (Robertson et al. 1982).

*Bacteroides* species are frequent isolates from periodontally involved sites and, in addition, have been implicated in a wide variety of soft tissue infections (Slots 1979; MacDonald 1962; Takazoe et al. 1971; Bartlett and Finegold 1972; Gorbach and
Bartlett 1974; Socransky 1977; Sutter, Citron and Finegold 1980; Slots 1982, Zambon et al. 1981, White and Mayrand 1981). As can be seen in Table I, **Bacteroides** may be unique among procaryotes in that it appears to possess a collagenase that remains associated with the cell.

Two major problems have frustrated past attempts to identify which procaryotes possess collagenolytic activity; one problem has been the uncertain taxonomic status of some organisms that appear to possess a collagenase. The second problem has been the use of widely varying methodologies to assess collagenolytic activity. Both of these problems have made data from different laboratories difficult to compare.

Collagenase activity may be assessed by a variety of methods. Radial diffusion of enzyme in a collagen matrix has been described (Yankielov et al. 1977) and the activity of the enzyme has also been measured by changes in optical rotary dispersion of the substrate (Keil et al. 1975). Collagenolysis may also be followed by measuring the reduction in viscosity of a collagen solution (Seifter and Harper 1970). Collagenolytic activity may be measured by the release of $^{14}$C-glycine containing peptides from reconstituted collagen fibrils, and this technique involves preparation and extraction of uniformly labelled collagen from animal tissues (Nagai et al. 1966). Birkedal-Hansen and Dano (1981) have described a collagen gel assay employing Type I collagen labelled *in vitro* using pyridoxal phosphate and
A soluble collagen assay has been described (Gisslow and McBride 1975) which utilizes as substrate collagen labelled \textit{in vitro} via acetylation with \textsuperscript{3}H- or \textsuperscript{14}C-acetic anhydride. Employing synthetic peptide scission alone as an index of collagenolysis is unreliable since enzymes that cleave the synthetic substrate, but not native collagen, have been isolated (Harper and Gross 1970). Collagenase reaction products may be visualized by polyacrylamide gel electrophoresis (Sahai and Gross 1967). A review and evaluation of many collagenase assay methods has appeared recently (Harris and Vater 1980).

**Microbial Mechanisms in the Pathogenesis of Periodontal Disease**

It has been suggested that soluble plaque factors, elaborated by bacteria which colonize the root surface play a role in initiation and progression of periodontal tissue breakdown (MacDonald and Gibbons 1962; Rizzo 1970; Sussman et al. 1969; Loesche 1976; Ranney 1978). Bacterial enzymes, including collagenases, are among these soluble plaque factors. If \textit{Bacteroides} collagenase, which appears to be cell-associated, plays a role in collagen degradation in periodontal diseases, then the enzyme must interact with host collagen. This could occur either by diffusion of soluble collagenase or by direct interaction of intact \textit{Bacteroides} cells with host collagen. If the collagenase remains cell associated throughout the growth cycle, it may be released in soluble form upon autolysis. If \textit{Bacteroides} cells use collagen peptides as a protein source, then a mechanism for maintaining these cells proximal to host collagen may function to
facilitate colonization by these organisms within the periodontal pocket.

Host cells produce attachment proteins (also called adhesive proteins and connective tissue matrix proteins) which may be extremely important in mediating a number of biologically significant attachment reactions. These include cell-cell interactions in host tissue, attachment of tissue culture cells to substrates, attachment of basal epithelial cells to basement membrane and attachment of fibroblasts to connective tissue components (Kleinman et al. 1981; Johnansson et al. 1981; Burrill et al. 1981). Fibronectin is a well characterized, high molecular weight, multifunctional adhesive glycoprotein found on cell surfaces, in extracellular matrices, and in serum and saliva (Simpson et al. 1982; Akiyama et al. 1981). It is produced by a variety of cell types and is able to bind cell membranes, collagen, gelatin, DNA, actin and a variety of connective tissue proteins (Kleinman et al. 1981). Laminin is a less well characterized glycoprotein synthesized by a variety of cells in culture and was first described by Timpl et al. (1979). It is a component of basement membranes and apparently enhances attachment of epithelial cells to Type IV collagen (Burrill et al. 1981). A few studies have appeared suggesting that some bacteria bind one or both of these proteins. Kuusela (1978) reported that fibronectin binds to Staphylococcus aureus. Binding between S. aureus and fibronectin has been characterized by Proctor et al. (1982). Recently, binding has been demonstrated to occur between group A,
C and G streptococci and fibronectin (Switalski et al. 1982; Myhre and Kuusela 1983). In addition, it appears that strains of \textit{S. pyogenes} (Switalski et al. 1983) and a strain of \textit{E. coli} (Speziale et al. 1983) bind laminin. Fibrinogen, a plasma protein which is enzymatically converted to fibrin in thrombosis appears to bind some pathogenic staphylococci and Group A, C and G streptococci (Hawiger, Hammond and Timmons 1975; Runehagen et al. 1981; Hawiger et al. 1982). Thus, attachment proteins may serve as "linker" molecules, attaching bacteria to host tissue cells or components. At least one organism, \textit{Staphylococcus aureus}, appears to possess a specific receptor for fibronectin (Esperson and Clemmenson 1982, Ryden et al. 1983). Host factors which may be important in modulating colonization of the periodontal pocket by oral microorganisms are unknown. No information is available concerning the interactions between \textit{Bacteroides} cells and host connective tissue matrix proteins. Such interactions might mediate collagenolysis as well as tissue colonization.

\textbf{SUMMARY}

While the role of oral microorganisms in the breakdown of host connective tissue in periodontal disease is not well understood, current evidence supports the following conclusions:

1. Inflammatory periodontal diseases are caused by oral microorganisms. Furthermore, a growing body of evidence suggests that different periodontal diseases have specific microbial eti-
ologies.

2. Loss of collagen from periodontal tissues is a prominent feature of periodontal disease.

3. Both host cells and selected oral microorganisms possess enzymes capable of degrading collagen. The relative importance of these two systems in eliciting periodontal breakdown is unknown.

4. *Bacteroides* species may be unique in its possession of a cell-associated collagenase. The significance of this cell-associated enzyme in an organism known to be predominant in periodontal disease is unknown.

5. Bacteria can attach to host cells and host cell components by a variety of mechanisms. The possible significance of these interactions in periodontal pathogenesis is essentially unexplored.
General Objective

The general objective of this research was to survey species of *Bacteroides* for their ability to degrade undenatured collagen under physiological conditions of pH and temperature. It was hypothesized that cell-associated collagenolysis is a unique feature of *Bacteroides* species which may be critical to this organism's role in pathogenesis of periodontal diseases. Examination of this hypothesis required assessment of several representatives of the oral microflora for collagenolytic activity. Knowledge of localization of cell-associated collagenase in *Bacteroides* species would be required in order to elucidate mechanisms by which these organisms may mediate collagenolysis in periodontal disease.
Specific Objectives

1) Survey selected species of the oral microflora, with particular emphasis on species of *Bacteroides*, for the presence of collagenolytic activity.

2) Define inhibition characteristics relative to other microbial collagenolytic activities.

3) Determine the localization of collagenolytic activity in selected *Bacteroides* species.

4) Initiate studies on the binding of *Bacteroides* species to host tissue attachment proteins.
Methods and Materials

All chemicals used were of reagent grade and obtained from commercial sources. All substrates and cofactors for assay of malate dehydrogenase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and phosphoglucose isomerase were obtained from Sigma Chemical, St. Louis, Mo. $^3$H-acetic anhydride and $^{14}$C-glycine were obtained from New England Nuclear, Boston, Mass. Trypsin and Clostridium histolyticum collagenase A, DNase I (bovine pancreas) and RNase (bovine pancreas) were obtained from Worthington, Freehold, N.J. $^{125}$I-labelled and unlabelled laminin and fibronectin were the gift of Dr. Magnus Höök, University of Alabama in Birmingham. $^{125}$I-labelling was accomplished by the chloramine T method (Hunter, 1978).

1. Growth of Bacteria

Bacteroides species were grown anaerobically at 37°C in basal anaerobic broth (BAB) with the composition given below.

**Basal Anaerobic Broth**

<table>
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<tr>
<th>Component</th>
<th>per liter</th>
</tr>
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<tbody>
<tr>
<td>Tryptone/Peptone</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.9 gm</td>
</tr>
<tr>
<td>Potassium phosphate, dibasic</td>
<td>0.45 gm</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.90 gm</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.90 gm</td>
</tr>
<tr>
<td>Potassium phosphate, monohydrate</td>
<td>0.45 gm</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.19 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Hemin</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.2 gm</td>
</tr>
</tbody>
</table>
*Bacteroides* species used in this study included seven strains isolated by Dr. Kenneth Kornman and two ATCC strains. Cell morphology and homogeneity for all organisms were confirmed by light microscopic examination. Organisms were characterized as to Gram-stain and morphology, acid production from glucose, esculin hydrolysis, nitrate and nitrite reduction, catalase activity, indole and ammonia production, urease and oxidase activity, and acid end-products detectable by gas-liquid chromatography. Identification of organisms was confirmed by computer-assisted classification schemes derived from Holdeman, Cato and Moore (1977), Holmberg and Nord (1975), Duerden, Holbrook and Collee (1977), and Kornman and Loesche (1980). Cultures were terminated in both log and stationary phases.

2. Harvesting of Bacteria

At the termination of growth, cells were collected by centrifugation at 3000 X g for 20 minutes. Media was saved and stored frozen as required and cells were washed in one of four different buffers as described below.

Bacteria to be assessed for collagenolytic activity were washed twice in Tris buffer (50mM) containing 5mM CaCl₂, pH 7.6 (Buffer A). Bacteria used in cell fractionation, osmotic shock and spheroplast experiments were washed three times in 0.01M Tris buffer, pH 8.0 (Buffer B).

Bacteria prepared for sucrose density gradient sedimentation velocity centrifugation were washed twice in 0.5M NaH₂PO₄ buffer containing 0.15M NaCl, pH 7.4 (Buffer C).
Bacteria used in binding studies with $^{125}$I-proteins were washed twice with phosphate buffered saline (0.14M NaCl, 0.02M sodium phosphate buffer, pH 7.4) containing 0.02% sodium azide (PBS-azide). Collagenolytic activity was not detectable in cells washed with PBS-azide.

3. Disruption of Bacteria

a. Assessment of Collagenolytic Activity

One gram (wet weight) of washed bacteria was suspended in 10 ml of Buffer A and sonicated in a water cooled vessel with a Branson W185 Cell Disruptor using three minute bursts of 20,000 Hz until microscopic examination of aliquots revealed essentially complete disruption, usually between nine and twelve minutes. All sonic extracts were centrifuged at 12,000 X g for 30 minutes. Collagenolytic activity was assessed both in supernatant and pellet fractions.

b. Comparison of Disruption Methods

Two grams (wet weight) of washed bacteria were suspended in Buffer B and subjected to:

**French Press Disruption**: bacterial suspensions were passed three times through a French press at 14,000 psi.

**Sonication**: bacterial suspensions received three 3-minute bursts at 20,000 Hz from a Branson W185 Cell Disruptor.

**Toluene Treatment**: bacterial suspensions were brought to 5% (v/v) toluene and incubated 45 minutes at 37°C with shaking (Wilkins 1972).
Extracts were centrifuged at 3000 X g for 20 minutes and yielded supernatant and pellet fractions. Pellets were washed once in Buffer B and resuspended in 5 ml of Buffer B. Washed whole cells (Buffer B) were used as controls.

4. Localization Studies

a. Preparation of low speed supernatant fraction for sedimentation velocity centrifugation.

Bacterial suspensions (1.5 gm wet weight/ml) were prepared in Buffer C containing 0.001M EDTA. Cells were disrupted by French press (3 X 14000 psi) and DNase (1 mg/gm wet weight of cells) and RNase (1 mg/gm wet weight of cells) were added and the French press extract was allowed to stand for 30 minutes at room temperature. The nuclease treated extract was centrifuged at 3000 X g for 20 minutes, the supernatant was decanted by pipette, and the supernatant was again centrifuged at 3000 X g. This low speed supernatant fraction was then subjected to sedimentation velocity centrifugation.

b. Preparation of Sucrose Gradients

Discontinuous sucrose gradients were prepared in cellulose nitrate tubes (40 ml capacity) using 60% sucrose, 45% or 40% sucrose, and 20% sucrose. All sucrose solutions were prepared in Buffer C. After layering sucrose solutions, gradients were allowed to equilibrate in the cold for two hours prior to use.

c. Sedimentation Velocity Centrifugation

French press low speed supernatant (1-3 ml) was layered on a prepared discontinuous sucrose gradient and centrifuged for 3
hours or overnight at 100,000 X g. Each gradient was collected from the tube bottom, passed through an LKB ultraviolet spectrophotometer and fractionated in 1 ml fractions. An LKB recorder plotted OD$_{280}$ as a function of fraction number.

d. Electron Microscopy of Gradient

The main OD$_{280}$ absorbing fractions corresponding to the outer membrane band were pooled and examined electron microscopically according to the method of Woo, Holt and Leadbetter (1979). The same procedure was followed for fractions corresponding to the inner membrane band and the soluble fraction of the cell. Pooled material was fixed using glutaraldehyde in cacodylate buffer. Fixed material was washed twice in cacodylate buffer and postfixed in OsO$_4$. This material was dehydrated in a graded series of ethyl alcohol, followed by propylene oxide and then embedded in Epon 812. Ultrathin sections were cut on a Porter-Blum Sorvall Model MT-2 ultramicrotome with a diamond knife. Sections were mounted on uncoated 300-mesh grids and stained with uranyl acetate and lead citrate. Sections were examined in a Phillips EM-200 electron microscope operating at 60 KV. The images were recorded on Kodak fine-grain release positive film no. 5302.

e. Osmotic Shock

The cells used in these studies were grown to either log or stationary phase and harvested by centrifugation. Cells were washed three times with cold 0.01M Tris–HCl pH 8.0, and collected by centrifugation. One to two grams (wet weight) of the pellet
was resuspended in 80 ml of one of the following solutions:

(a) 0.03M Tris + 20% (w/v) sucrose, pH 8.0
(b) 0.03M Tris + 20% (w/v) sucrose + 3mM EDTA, pH 8.0
(c) 0.03M Tris + 20% (w/v) sucrose + 30mM CaCl₂, pH 8.0
(d) 0.01M Tris + 20% (w/v) sucrose + 1mM EDTA + 1mM sodium ascorbate, pH 7.2

Cells were equilibrated in one of the above buffers for 10 minutes in the cold with gentle stirring, and collected by centrifugation. Osmotic shock was performed as described by Neu and Heppel (1965). The cells were resuspended in a small volume of the appropriate Tris-sucrose solution and rapidly added to 90 ml of cold distilled water with stirring. The cells were collected by centrifugation and the pellet was resuspended in 3-5 ml of Buffer C. Resuspended pellets were disrupted by french press and these pellets, Tris-sucrose supernatant and the cold water wash were assessed for collagenolytic activity, malate dehydrogenase activity, alkaline phosphatase activity and protein content.

f. Spheroplasting

Spheroplasting of Bacteroides intermedius was attempted as described by Neu and Heppel (1964). Two grams (wet weight) of cells were washed three times (Buffer B) and resuspended in 100 ml of 0.03M Tris + 20% sucrose (w/v), pH 8.0 to which was added EDTA (final concentration 1mM) and lysozyme (final concentration 1 mg/ml). This mixture was incubated at 37°C. At selected times (1 hour and 2 hours) portions of the suspension were centrifuged at 12000 X g for 10 minutes. The supernatant fractions were
saved and the pellets were resuspended in 100 ml of distilled water to lyse the spheroplasts. Unbroken cells were removed by centrifugation at 12000 X g for 10 minutes and spheroplast lysates (supernatants) were saved. Resuspended pellets (distilled water), Tris-sucrose supernatants and spheroplast lysates were examined for collagenolytic activity, MDH activity, alkaline phosphatase activity and protein content.

5. Enzyme Assays
   
a. Collagenase

Quantitative measures of collagenolytic activity included the $^{14}$C-labelled radiofibril gel assay as described by Robertson, Taylor and Fullmer (1972) and $^3$H-acetylated collagen assay by a modification of the method of Gisslow and McBride (1975).

For the radiofibril assay, $^{14}$C-glycine-labelled chick calvaria collagen in 0.5M acetic acid (Siegal and Martin 1970) is added to acid extracted rat tail collagen (4 mg/ml, 0.5M acetic acid) prepared as described by Bornstein and Piez (1966), in an amount sufficient to yield approximately 50,000 cpm/ml and exhaustively dialyzed against the collagenase assay buffer (5 mM CaCl$_2$, 200 mM NaCl, 30 mM Tris, pH 7.5). The preparation is highly resistant to nonspecific protease digestion and reaction mixtures containing 0.5 ug trypsin/ug collagen rarely exceed 6% digestion. Aliquots of the collagen mixture are added to 1.5 ml polycarbonate centrifuge tubes. The buffered collagen forms gels promptly upon heating to 36.5°C in a water bath. After addition of enzyme or buffer controls, all assays are incubated 36.5°C for
1 to 20 hours and the subsequent reaction mixture is centrifuged. An aliquot of the supernatant is counted by liquid scintillation spectrometry.

For soluble collagen assays, the collagen substrate (Type I rat tail collagen) in 0.1% acetic acid, 40°C, is brought to pH 8.0 by addition of 1.0M K$_2$HPO$_4$. Five millicuries (10.2 mg) of H$^3$-acetic anhydride in 1.5 ml benzene is added drop-wise to the constantly stirred collagen solution over a period of 3 hours. The reaction mixture is adjusted to pH 4.0 with glacial acetic acid, made 5% with respect to NaCl to precipitate the collagen and centrifuged. The collagen pellet is washed, redissolved in 0.1% acetic acid, dialyzed exhaustively against 0.1% acetic acid and lyophilized. The collagen substrate is redissolved in 0.1% acetic acid at a concentration of 1 mg/ml (10,000-15,000 cpm/50 ul) and stored frozen. Collagenase assays consist of 1-20 hour incubation at 25°C or 37°C of 0.1 ml $^3$H-collagen solution, 0.1 ml assay buffer, and 0.1 ml test agent. After incubation, two tenths milliliter of a solution containing equal parts of 0.4 N phosphotungstic acid and 2.0 N HCl is added to the reaction mixture, the samples are centrifuged and 0.1 ml aliquots of the supernatant are counted by liquid scintillation spectrometry.

Sodium azide (0.05%) is included in the assay buffer in both methods. Assay controls include purified preparations of Clostridium collagenase, trypsin (0.5 ug/ug collagen), and assay buffer alone.

b. Other Enzymes
Malate dehydrogenase activity was determined as described by Ochoa (1955). Activity determination is based upon measurement of the rate of oxidation of NADH (decrease in optical density at 340 nm) in the presence of enzyme and excess oxalacetate.

Alkaline phosphatase activity was determined essentially as described by Wilkins (1972). The sample (0.5 ml) is mixed with 2.0 ml of phosphatase substrate solution (1 mg/ml p-nitrophenyl phosphate in 1.0M Tris buffer, pH 8.5) at 25°C. Activity is measured as the increase in optical density at 410 nm per minute.

Glucose-6-phosphate dehydrogenase activity is determined by the method of Kornberg and Horecker (1955). Briefly, NADH reduction is followed by observing the increase in optical density at 340 nm, in the presence of enzyme and excess glucose-6-phosphate. Phosphoglucoisomerase activity is determined colorimetrically by the method of Slein (1955) and is based on the appearance of fructose-6-phosphate from an excess of glucose-6-phosphate. Lactate dehydrogenase is determined by the method of Kornberg (1955). Activity is determined by measuring the decrease in optical density at 340 nm (oxidation of NADH) as pyruvate is converted to lactate. Protein was determined using the Bio-Rad Protein Assay (Bradford 1976).

All organisms used in localization studies were also characterized enzymatically using the API ZYM system. Cells were grown to stationary phase and assays were performed aerobically as described by Laughon et al. (1982).

6. SDS polyacrylamide disc gel electrophoresis
SDS polyacrylamide disc gel electrophoresis was performed essentially as described by Furthmayr and Timpl (1971) with the exception that all solutions used and sample preparation were as described in LKB application Note 306. The phosphate buffer system was used as described and electrophoresis was accomplished using 5% gels. Gels were prepared in 75 mm X 5 mm (inside diameter) tubes and 10 ul samples in 100 ul of 50% sucrose containing beta-mercaptoethanol (10 ul) and bromphenol blue solution (10 ul) were applied to the tubes following 30 minutes of pre-electrophoresis. Electrophoresis was performed at 5°C using a Buchler Polyanalyst Electrophoresis Apparatus with an eighteen tube capacity. Runs were performed at constant current (6 mA/tube). At the conclusion of the run, gels were removed from the tubes and fixed, stained and destained as described (LKB Application Note 306).

7. Binding Studies

Binding studies were performed as described by Switalski et al. (1982), and performed, in part, in the laboratories and with the gracious assistance of Dr. Magnus Höök, Diabetes Hospital, University of Alabama in Birmingham. Bacteria were harvested during log or stationary phase by centrifugation at 1300 X g for 20 minutes and washed twice with PBS-azide (0.14M sodium chloride, 0.02% sodium azide, 10 mM phosphate buffer, pH 7.4). Bacteria were resuspended in PBS-azide, counted in a Petroff-Hauser counting chamber and adjusted to a concentration of 1 x 10^10/ml.
a. Binding of $[^{125}\text{I}]$ protein to bacteria

Binding of $^{125}\text{I}$-protein to bacteria was assessed as described by Switalski et al. (1982). A known number of bacteria (approximately $10^9$) were incubated with $2 \times 10^4$-$10^5$ cpm of $^{125}\text{I}$ protein in a total volume of 0.6 ml PBS containing 0.1% bovine serum albumin. All binding studies were performed at room temperature ($24^\circ\text{C}$). Incubation tubes were precoated with PBS-albumin, and tubes were incubated end-over-end. At the end of the incubation period, 200 ul of the mixture was carefully added to 0.5 ml of PBS layered on top of 3 ml of Percoll (density = 1.020 g/ml) in PBS. The samples were centrifuged in a swinging bucket rotor at 1300 X g for 20 minutes. During centrifugation, bacteria with attached labelled proteins sediment through the Percoll and form a pellet at the tip of the tube, whereas the incubation mixture, containing unbound labelled protein, remains on top of the Percoll.

The supernatants were aspirated and the radioactivity associated with the pellet was quantitated in a gamma counter. Binding assays were always run in duplicate. In the event that bacteria did not sediment through Percoll, the assay was modified as follows: After incubation, an aliquot of incubation mixture was removed and placed in a precoated tube. Three ml of PBS were added to the tube and the samples centrifuged as above. The pellet was resuspended in 0.5 ml of PBS and then 2.5 ml of PBS was added to the tube. The tubes were again centrifuged as above. After the second centrifugation, the radioactivity
present in the pellet was quantitated.

b. Time Dependence, Reversibility and Saturability of Binding of $^{125}$I-Laminin to \textit{Bacteroides gingivalis} (W)

Incubation of $^{125}$I-laminin with a constant number of bacteria was conducted until no further increase in levels of bound laminin was observed. Samples were taken at appropriate times and radioactivity associated with bacteria was determined as described above.

Reversibility of binding was assessed by attempting to displace bound laminin with a large excess of unlabelled laminin. Bacteria were incubated with $^{125}$I-laminin as described above. At an appropriate time, a 1000 fold excess of unlabelled laminin was added. The bacteria were again incubated for an appropriate time end-over-end. Radioactivity associated with the bacteria was determined as described above.

If the number of binding sites on the cell surface for a given protein is limited, then it should be possible to saturate these sites. The assay was used to examine this possibility by incubating a fixed number of cells ($10^9$) with an increasing amount of radiolabelled protein of known specific activity. Bacteria were incubated with $^{125}$I-laminin of known specific activity for an appropriate time and radioactivity associated with bacteria was determined as described above for each concentration of laminin used.

c. Release of laminin binding activity from bacteria

\textit{Bacteroides gingivalis} W cells were digested with trypsin in
an attempt to release active laminin binding activity (Switalski et al. 1982). Cells were grown to stationary phase and harvested as described above. Bacteria were resuspended in PBS-azide at 1 x 10^{10} cells/ml and heated at 56^\circ C for 20 minutes. Cells were collected by centrifugation and resuspended in PBS without azide at a concentration of 2.5 x 10^{10}/ml. The cells were then digested with trypsin (25ug/ml) for 30 minutes at 37^\circ C. The reaction was stopped with an excess of soybean trypsin inhibitor (50ug/ml). The trypsin treated cells were collected by centrifugation, washed once with PBS and resuspended in PBS to a final concentration of 1 x 10^{10}/ml. Material released from bacteria during incubation with trypsin was used in inhibition experiments, replacing appropriate quantities of PBS-albumin in the incubation mixture. Binding of laminin to trypsinized cells was determined directly.

8. Statistical Treatment of Data

Multiple trials were performed for each experiment as noted. For experiments where triplicate samples were used, means and standard deviations were calculated. For experiments where duplicate samples were used, variability of the data was expressed by calculating the mean and standard deviation of the difference between duplicate samples.
Results

I. Collagenolysis by *Bacteroides* species

Essentially identical results were obtained when collagenolysis was assessed using the soluble collagen assay and the assay employing reconstituted collagen fibrils as substrate. Hence, all values for collagenolytic activity shown are based on the former. All tested species of *Bacteroides* (nine strains representing seven species) demonstrated collagenolytic activity. Relative percent collagenolysis by media and cell sonic supernatants from each organism harvested in late stationary phase is shown in Table 2. Organisms which do not show collagenolytic activity under the same conditions are described in Robertson et al. (1982) and include species of *Capnocytophaga*, *Fusobacterium Selenomonas* and *Actinomyces*.

The effect upon *Bacteroides* collagenases of exposure to serum, EDTA and boiling are shown in Table 3. Prior incubation of sonic supernatant with serum (37°C for 30 min.) at a final dilution of 1:4 inhibited *Bacteroides* enzyme activity by 22% to 70%, compared to incubation of sonic supernatant in buffer alone. Serum alone at this dilution showed no collagenolytic activity. Prior boiling of sonic supernatant or assay of collagenolytic activity in the presence of EDTA reduced collagenolysis to levels approaching those obtained with trypsin. Incubation with cysteine or dithiothreitol had no effect on the percent collagenolysis observed.

Acrylamide gel electrophoresis of the collagen substrate,
**Bacteroides gingivalis** (1416.10) sonic supernatant, and collagen-sonic supernatant mixtures at zero time and 3 hr post incubation at 37°C is shown in Figure 1. Substantial collagen degradation at 3 hr was observed. The electrophoretic patterns confirmed results obtained with the 14C-labelled collagen assays and suggested that the microbial enzymes caused multiple scission of the collagen molecule. The sonic supernatants are clearly crude preparations, and the degradation observed is probably the result of both collagenase and neutral protease activity.

A. Kinetics of Collagenolysis

Assessing the effect of increasing concentrations of substrate on the rate of collagenolysis by a fixed amount of enzyme was technically impractical for both the soluble collagen assay and the radiofibril assay. In the case of the soluble collagen assay, increasing the concentration of collagen over even a small range resulted in unmanagably viscous solutions. This technical problem is discussed by Gisslow and McBride (1975). In the case of the radiofibril assay, the properties of the gel were altered by changing ratios of buffer to collagen, and gelation failed to occur unless adequate buffer was added to raise the pH above 7.2 (at 37°C). Thus, the collagen concentration of the gel could not be varied except over a very narrow range. This difficulty was also encountered by Harris and Vater (1980) using a gel assay.

This limitation of the assay techniques was significant in only a few experiments in which quantitative assessment of the percent of total collagenolytic activity in a given fraction was
necessary. In these experiments, a variety of enzyme concentrations was assayed using a constant concentration of substrate. Experiments in which calculation of the relative amount of collagenolytic activity present was necessary, were performed under conditions where the rate of collagenolysis depended only upon the concentration of enzyme present.

1. Time Dependence

The percent collagen solubilized as a function of time for the sonic supernatant of *B. gingivalis* (W) is shown in Figure 2. The reaction appears to be linear over a 20 hour period at the enzyme concentration used.

2. Concentration Dependence

The collagen solubilized as a function of sonic supernatant concentration is shown in Figure 3 for extracts of *B. gingivalis* (W), *B. gingivalis* 1416.10, and purified *Clostridium histolyticum* collagenase. Reactions proceeded for 20 hours. As can be seen in Figure 3, doubling the extract concentration approximately doubled the amount of collagen solubilized (up to 50% extract) for *B. gingivalis* (W). The collagen solubilized by the *B. gingivalis* 1416.10 extract and the *C. histolyticum* enzyme increased as a function of the amount of enzyme added over the range of enzyme concentrations evaluated.

3. Temperature Dependence

Both the soluble collagen assay, as described by Gisslow and McBride (1975) and the reconstituted fibril (gel) assay, as described by Robertson, Taylor and Fullmer (1972) were routinely
performed at 37°C. Both assays have also been performed at 24°C using sonic extract supernatants of *Bacteroides intermedius* 794.01 and *Bacteroides gingivalis*(W). In the soluble assay both bacterial extracts gave similar results. The relative collagenolysis observed at 20 hours, 24°C was 50-60% of the value achieved in the identical assay performed at 37°C. In the gel assay, the relative collagenolysis by both *Bacteroides* extracts at 20 hours and 24°C was approximately equal to that seen at 37°C for the same time period. The trypsin sensitivity of both soluble collagen substrate and the gel substrate were equal at 24°C and 37°C. Neither collagen substrate appeared to be more sensitive to digestion by trypsin at 37°C than at 24°C. Collagenolysis by trypsin remained in the range of 3-8% for both collagen substrates at 24°C and 37°C.

B. Effect of Sonication on Recovery of Collagenolytic Activity

The effect of length of time of sonication on recovery of collagenolytic activity from *Bacteroides gingivalis* 1416.10 is shown in Figure 4. Cells were sonified as described in Methods. Three minute cycles of sonication (in an ice water bath) were interrupted by three minutes of cooling in an ice water bath. The data is presented in Figure 4 and suggests that collagenolytic activity recoverable in the low speed pellet (12,000g X 20 min.) continues to decrease as a function of length of time of sonication. Collagenolytic activity in the uncentrifuged sonic extract decreases somewhat continuously as a function of length of time of sonication and recovery of collagenolytic activity in
the sonic extract supernatant increases up to 12 minutes of sonication, after which recoverable activity decreases in the supernatant. These results suggest that sonication should not be performed for longer than 12 minutes (four three minute cycles). It appears that after this time there is a net loss of activity since activity lost by the pellet is not recovered in the supernatant fraction.

C. Api-zym profiles of *Bacteroides intermedius* and *Bacteroides gingivalis*.

The studies which follow focused on two organisms; *Bacteroides intermedius* 794.01 and *Bacteroides gingivalis (W)*. These organisms were characterized enzymatically using the API-ZYM system, as described in the Methods. *Bacteroides intermedius* 794.01 was positive for alkaline phosphatase, esterase, esterase lipase, acid phosphatase, phosphoamidase, and alpha-glucosidase. *Bacteroides gingivalis (W)* was positive for the same enzymes as *Bacteroides intermedius* 794.01 and in addition was positive for leucine aminopeptidase, trypsin and N-acetyl-beta-glucosaminidase. These results are consistent with those reported by Laughon et al. (1982) for these bacteria.

II. Fractionation Studies

Washed, undisrupted *Bacteroides intermedius* 794.01 incubated at 37°C for 30 minutes) were compared with *Bacteroides intermedius* 794.01 disrupted by french press, sonication and toluene treatment for recovery of protein, collagenolytic, alkaline phosphatase and malate dehydrogenase activities. Alkaline
phosphatase was assessed because it is present in *Bacteroides* and because it has a periplasmic location in many gram negative bacteria (Heppel 1967). Malate dehydrogenase was assessed as a cytoplasmic marker enzyme. Attempts were made to assay glucose-6-phosphate dehydrogenase and phosphoglucoisomerase activities in these bacteria but these activities were not detectable. Total protein recovered in uncentrifuged extracts was identical for all three methods of disruption and the same as that demonstrated in an aliquot of control, undisrupted cells. Total collagenolytic activity was greatest in whole undisrupted cells. Uncentrifuged French press extracts and sonic extracts retained an average of 75% of the activity of the controls and toluene treated extracts retained an average of 90% of the activity of the controls. French press extracts and sonic extracts had the same alkaline phosphatase activity as the controls, whereas toluene treated cells retained an average of 80% of the total activity. Malate dehydrogenase activity was undetectable in sonic extracts and toluene extracts. Total malate dehydrogenase activity was similar for control cells and French press extracts. All three enzymes were assayable in whole undisrupted cells although MDH activity was detected after a short lag. The highest collagenolytic activity was exhibited by undisrupted cells.

The data presented for control cells (Table 4) suggests that whole cells incubated in buffer for short periods do not leak protein or the enzymes examined, since following incubation (37°
C for 30 min.) and centrifugation (12,000 X g for 20 min.) essentially all protein and enzyme activity remain with the cells. Collagenolytic activity remains with cells following several weeks of refrigeration in buffer. Following French press disruption, an average of 87.9% the protein, 62.4% of the collagenolytic activity and essentially all of the alkaline phosphatase and malate dehydrogenase activity appeared in the supernatant fraction. Sonication released an average of 73.2% of the protein, 64.2% of the collagenolytic activity and essentially all of the alkaline phosphatase activity into the low speed supernatant fraction whereas toluene treatment released an average of only 11.1% of the protein, 23.4% of the collagenolytic activity and 38.6% of the alkaline phosphatase activity into the low speed supernatant fraction. These results suggest the French press disruption is an effective and useful method of cell disruption since it releases malate dehydrogenase and alkaline phosphatase activities quantitatively, and retains high levels of total collagenolytic, alkaline phosphatase and malate dehydrogenase activities. Hence, this method was used in the localization studies which follow.

III. Localization Studies

A. Sedimentation Velocity Centrifugation

A French press disrupted low speed supernatant fraction from Bacteroides intermedius 794.01 was layered on a discontinuous sucrose gradient and subjected to sedimentation velocity centrifugation as described in the Methods. The results are shown in
Figure 5. The profile was the same whether centrifugation was performed for three hours or overnight. Three protein peaks can be seen which are labelled I, II and III. Electron microscopic examination of these fractions has shown them to be an outer membrane rich fraction (I), an inner membrane rich fraction (II) and a soluble fraction (III). Figure 6 is an electron micrograph of the outer membrane rich fraction. The predominant fragments consist of cell wall and associated peptidoglycan. The inner membrane rich fraction (Figure 7) shows the predominance of single membrane bound vesicles characteristic of cytoplasmic membrane. Although small amounts of collagenolytic activity appear to be associated with the membrane fractions, essentially all of the collagenolytic, alkaline phosphatase and malate dehydrogenase activities appear to be recovered in the soluble fraction. Similar results were obtained for Bacteroides gingivalis (W). The results shown in Figure 5 suggest that under these conditions of cell disruption (0.001 M EDTA) only very small amounts of collagenolytic activity are associated with membranes. Essentially all of the collagenolytic activity appears to be associated with the soluble fraction of the cell along with alkaline phosphatase and malate dehydrogenase activities.

B. Osmotic Shock and Spheroplasting

Osmotic shock of Bacteroides gingivalis (W) and Bacteroides intermedius 794.01 was performed under a variety of conditions. All experiments in which stationary phase cells were used demonstrated excessive cell breakage, about 25% in the Tris-sucrose
solutions and about 25% in the cold water washes, as assessed by the amount of total protein and MDH activity present in these solutions relative to the cell pellet collected from the cold water wash. That is, 50% of the cells were lost to breakage during the entire procedure. The excessive breakage observed for stationary phase cells was independent of the Tris-sucrose solutions used and bacterial species examined, and probably reflects the prolonged pipetting and stirring required to resuspend the bacteria in the Tris-sucrose solutions and the cold water wash. Log phase cells resuspended more readily in Tris-sucrose solutions and cold water washes. However, the amount of cell breakage remained a problem, averaging 20-30% for the entire procedure. Minimal cell breakage (as assessed by release of protein only as MDH activity was too low to detect reliably) when dilute solutions of log phase cells (0.1g in 30 ml) equilibrated with ascorbate (1mM sodium ascorbate present in all solutions except the cold water wash) were employed. Results of a representative experiment using Bacteroides intermedius 794.01 are shown in Table 5. Although it is not possible to adequately assess cell breakage since MDH activity could not be reliably determined at this cell concentration, the rather small release of protein (an average of 2.8% of total) and the rather large release of alkaline phosphatase (37.5% of total) and collagenolytic activity (50.5% of total) into the cold water wash suggests that collagenolytic activity and alkaline phosphatase activity may have a periplasmic location in Bacteroides intermedius (794.01).
Attempts to produce spheroplasts using EDTA (1mM) and lysozyme (1 mg/ml) as described in Methods were unsuccessful. Using *B. intermedius*, excessive cell breakage (as assessed by protein and MDH activity) rather than spheroplast formation occurred during the incubations with EDTA (1mM) and lysozyme (1 mg/ml).

IV. Binding Studies

The observation that *Bacteroides* species do not release collagenolytic activity into media during growth (except perhaps by autolysis during late stationary phase), coupled with the observation that whole cells appear to be able to carry out collagenolysis suggested that either these cells internalize native collagen or that collagenolysis occurs at the cell surface. If collagenolysis is carried out at the bacterial surface then perhaps, in situ, these bacteria require a location proximal to collagen in order for collagenolysis to occur. A molecule capable of "linking" *Bacteroides* to collagen may function to maintain the organism proximal to this peptide source. The attachment proteins fibronectin and laminin appear able to bind both collagen and some bacteria (Switalski et al. 1982; Kleinman et al. 1981; Burrill et al. 1981). If fibronectin or laminin could serve as "linker" molecules between *Bacteroides* and collagen then *Bacteroides* species might be expected to bind fibronectin and/or laminin. We have examined the ability of *Bacteroides gingivalis* W to bind $^{125}$I-laminin and $^{125}$I-fibronectin and have found that this organism binds $^{125}$I-laminin but
does not appear to bind $^{125}$I-fibronectin under the experimental conditions described in the Methods. Two strains of *Bacteroides capillus* (928.05 and 938.11) failed to bind $^{125}$I-laminin under the same conditions.

A. Binding of $^{125}$I-laminin to *Bacteroides gingivalis* (W)

1. Time Dependence

The time dependence of the binding of $^{125}$I-laminin to *Bacteroides gingivalis* (W) for log phase cells and stationary phase cells is shown in Figure 8. The results indicate that binding was rapid for both log and stationary phase cells. Log phase cells bound most of the laminin (binding was 70% complete) within the first 15 minutes. However, the amount of laminin bound continued to increase for the first hour, after which time no further increase in laminin binding occurred. Stationary phase cells achieved maximal binding within the first 15 minutes after which time no further increase in laminin binding was observed. In addition, it appears that stationary phase cells bound slightly more $^{125}$I-laminin (9.3% of radioactivity added/10$^9$ cells) than did log phase cells (6.0% of radioactivity added /10$^9$ cells). In the following experiments, binding assay incubations were carried out for 1 hour when log phase cells were studied, and for 15 minutes when stationary phase cells were studied.

2. Reversibility Of Binding

Log phase cells were incubated with $^{125}$I-laminin for one hour, at which time a large excess (1000 fold or greater) of unlabelled laminin was added in an attempt to displace bound
laminin from bacteria. The mixture was incubated for an additional hour. The unlabelled laminin failed to displace bound $^{125}\text{I}$-laminin and thus it appears that binding of $^{125}\text{I}$-laminin to bacteria is functionally irreversible. Attempts were made to displace $^{125}\text{I}$-laminin with large excesses of other proteins including fibrinogen, fibronectin, IgG (human), albumin, fetuin and alpha-acid glycoprotein. None of these proteins was able to displace $^{125}\text{I}$-laminin from the bacteria. When reversibility was assessed for $^{125}\text{I}$-laminin bound to stationary phase cells, the bacteria clumped almost immediately upon addition of a large excess of unlabelled laminin. This clumping phenomenon was not observed with log phase cells, nor was visible clumping observed for stationary phase cells at the low concentrations (picogram range) of $^{125}\text{I}$-laminin used to examine time dependence. Fine clumps were noted visually at laminin concentrations as low as $10^{-3}$ ug/ml.

3. Saturation Kinetics

The saturation curve for binding of laminin to log phase Bacteroides gingivalis (W) is shown in Figure 9. Increasing amounts of $^{125}\text{I}$-laminin (5 ug to 100 ug, specific activity $1.8 \times 10^4$ cpn/ug) were added to tubes containing bacteria ($6.7 \times 10^8$) and incubation was carried out for one hour. Samples were assessed for bound $^{125}\text{I}$-laminin as previously described. The amounts of $^{125}\text{I}$-laminin bound to the bacteria increased in proportion to the amounts of radiolabelled protein added to the incubation mixture. However, additions greater than approximately 70
ug/sample did not result in a further increase in $^{125}$I-laminin bound to bacteria. The number of laminin binding sites present on the cell surface is about 1500, assuming a molecular weight of 900,000 for laminin and assuming that all available binding sites are saturated at concentrations of added laminin above 70 ug (that is $6.7 \times 10^8$ bacteria bind 1.5 ug laminin at saturation).

A Scatchard analysis (Scatchard 1949) of the saturation curve is included in Figure 9 (Insert) for purposes of discussion. The slope was used to calculate an apparent association constant, $K_a = 1.28 \times 10^{10} M$.

Saturation data for binding of stationary phase cells was also collected but complicated by visible clumping of bacteria. It was of some concern that unbound $^{125}$I-laminin might be nonspecifically included in clumps. When such clumping occurred, the saturation curve was biphasic and saturation appeared to be reached at 70 ug laminin added.

4. Release of Laminin Binding Activity

Functional laminin receptors have been released from $E. coli$ by trypsin treatment (Switalski et al. 1983) and these results prompted similar studies with $Bacteroides gingivalis$ (W). Trypsin treatment of cells was performed as described in the Methods. Material released by trypsin treatment of $B. gingivalis$ (W) was found to inhibit binding of $^{125}$I-laminin to untreated cells and the inhibition curve is shown in Figure 10. Material released from bacteria by trypsin ("trypsinate") was used
directly in inhibition experiments and Figure 10 represents the effect of adding increasing amounts of trypsinate (replacing PBS-albumin in the binding assay incubation mixture) to untreated stationary phase \textit{B. gingivalis} (W). Trypsin treated cells bound only one-half as much $^{125}$I-laminin as did untreated cells.
Collagenolysis by *Bacteroides* species

The results of this study are consistent with previous reports that certain members of the oral microflora elaborate a collagenase which may contribute to periodontal connective tissue degradation. Collagenolytic activity was demonstrated in all tested species of *Bacteroides*. Differences in collagenolytic activity from that reported by Steffen and Hentges (1981) for *B. fragilis* and *B. thetaiotaomicron* and by Mayrand et al. (1980) for *B. melaninogenicus* may be related to enzyme extraction method, methods of assay, and strain specificity. The importance of strain specificity is compatible with the considerable variation reported by Steffen and Hentges (1981) in elaboration of other hydrolytic enzymes among strains of *B. fragilis* and *B. melaninogenicus*. Similarly, isolates of *B. melaninogenicus* tested by Mayrand et al. (1980) could be divided into two groups. The first group possessed a cell associated protease active against both Azocoll and casein and the second group was not active against either substrate.

Acrylamide gel electrophoresis patterns suggest that the *Bacteroides* preparations effect multiple scissions of the collagen molecule as opposed to vertebrate collagenases which cleave undenatured collagen at a single peptide bond. These observations must be tempered by the fact that *Bacteroides* extracts were crude preparations and presumably also contained a variety of neutral proteases. *B. gingivalis* (W) possesses trypsin activity
as verified by the API ZYM system. This observation is consistent with the findings of Laughon et al. (1982). It is clear, however that Bacteroides species can cause extensive degradation of the collagen molecule, an ability which is not shared under the same conditions by other members of the indigenous oral flora including species of Fusobacterium, Actinomyces, Capnocytophaga, and Selenomonas (Robertson et al. 1982).

The Bacteroides collagenolytic activity is heat labile and inhibited by serum and EDTA. These characteristics are shared by other microorganisms reported to elaborate multiple scission enzymes including Clostridium histolyticum, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Achromobacter iophas, Vibrio species, and Bacillus cereus (Waldvogel & Swartz 1969, Welton & Woods 1975, Kiel-Dlouha 1976, Carrick & Berk 1975, Merkel, Dreisback & Ziegler 1975, Soderling & Paunio 1977, Mandl, MacLennon & Howes 1953). Unlike collagenases from various human tissues which appear to share both collagen cleavage site and immunologic identity (Werb & Reynolds 1975, Harris and Cartwright 1977), specific collagenases from different organisms appear to be dissimilar in electrophoretic mobility, subunit structure, and substrate specificity. Immunologic identity has been demonstrated for Clostridium isozymes, but no serologic cross reactivity has been observed between Clostridium collagenase and the enzymes elaborated by Vibrio species or Acromobacter iophas (Welton & Woods 1975, Merkel & Dreisback 1978, Lwebuga-Mukasa, Harper & Taylor 1976).
Methods of Assessing Collagenolytic Activity

In most laboratories, specific collagenase activity is measured with soluble or fibrillar type I collagen as substrate (Harris and Vater 1980). In attempts to lower the detection limit and facilitate substrate preparation, production of native substrates with high specific radioactivity has been important (Birkedal-Hansen and Dano 1981). In this study both biosynthetically labelled collagen (gel assay) and chemically labelled collagen (soluble assay) have been employed as substrates.

Recently, Birkedal-Hansen and Dano (1981) have described a method for $^3$H-labelling Type I rat tail collagen using pyridoxal phosphate and $^3$H-borohydride and have achieved very high specific activity $^3$H-collagen. Their data suggests that native type I collagen, whether radiolabelled or not, is cleaved in the helical region by trypsin at subdenaturation temperatures. They have suggested that when collagenase assays are performed at temperatures close to the denaturation temperature of the collagen, reaction specificity is lost and collagen can be degraded by nonspecific proteases. These authors concluded that in order to remain specific, collagenase assays must be performed 10-12°C below the denaturation temperature of collagen, that is gel assays should be performed at 35-37°C (Tm of collagen = 47°C) and assays employing soluble collagen should be performed at 27-29°C (Tm of collagen = 39°C).
The results of the present study are in conflict with those reported by Birkedal-Hansen and Dano (1981). The results of the current study suggest that:

1) Type I collagen (rat tail) labelled using \(^3\text{H}\)-acetic anhydride, when used in a soluble collagen assay, is no more susceptible to digestion by trypsin at 37°C than at 24°C, although total collagenolysis by collagenase was greater at 37°C.

2) Chick calvaria collagen labelled biosynthetically with \(^{14}\text{C}\)-glycine, combined with type I rat tail collagen as carrier and used in a gel assay is no more susceptible to digestion by trypsin at 37°C than at 24°C. In addition, after 20 hours incubation with *Bacteroides* sonic extracts, total collagenolysis achieved at both temperatures was essentially the same.

The reason for the discrepancy between this data and that of Birkedal-Hansen and Dano (1981) is not clear. However, it appears that both the soluble and gel assays have been reliable indicators of collagenolysis as they have been employed in the current studies.

The ability of *Bacteroides* sonic extracts to degrade type I collagen gels at 24°C, (i.e. 23°C below the denaturation temperature of the collagen) suggests that these extracts possesses true collagenase activity.
Localization Studies

Gram-negative bacteria consist of a cytoplasm surrounded by three concentric envelope layers: an inner (cytoplasmic) membrane, a peptidoglycan cell wall, and an outer membrane. These layers define two cell compartments: the periplasm which consists of the inner and outer membranes and the aqueous space (periplasmic space) between these two membranes and the cytoplasm which is the aqueous compartment bounded by the inner membrane. According to Heppel (1967) there are several possibilities for localization of enzymes within gram negative bacteria. Enzymes may be localized on either the outer membrane, the inner membrane or within the periplasmic or cytoplasmic compartments. It also appears that some enzymes have multiple locations within the cell (Poirier and Holt 1983a).

Reliable methods of subcellular fractionation are required for the analysis of bacterial protein localization. Centrifugation at 100,000 X g allows the division of a bacterial lysate into two fractions, a pellet which is composed of the bacterial cell envelope containing the inner membrane, outer membrane and peptidoglycan and a supernatant containing cytoplasmic and periplasmic components. The degree of contamination of each fraction with the other is low when centrifugation is performed at this high centrifugal force (Silhavy et al. 1983).

Several methods for separating the two bacterial membranes can be employed. In general, whole cells or spheroplasts may be lysed osmotically, by sonication, French press or detergents.
Membranes are collected from the lysate by centrifugation (100,000 × g), washed and then separated into inner and outer membrane fractions by employing methods such as isopycnic centrifugation in a sucrose gradient, by electrophoresis or by selective detergent solublization (Silhavy et al. 1983). Once cells are broken, cytoplasmic components cannot be distinguished from periplasmic components because both are soluble. To separate the components of these two cellular compartments, one must remove or alter the outer membrane without disrupting the inner membrane. Usually this is accomplished by spheroplasting or controlled osmotic shock (Neu and Heppel 1965). These methods release periplasmic components, whereas cytoplasmic components remain associated with the cell.

All of these methods rely on a controlled disruption of the bacterial cell to yield fractions enriched for a specific cellular compartment. The conditions for this type of cellular fractionation have been empirically determined, generally employing *E. coli* as the prototype organism. Often these procedures must be modified when applied to other organisms (Poirier and Holt 1983a). There is virtually no information available concerning cell localization of degradative enzymes of *Bacteroides* species.

Cell associated degradative enzymes of many Gram-negative bacteria appear to be located external to the cytoplasmic membrane (Bhatti et al. 1976; Brockman and Heppel 1968; Chatterjee et al. 1976; Cheng et al. 1970a,b, 1971; Costerton 1970, 1973;
Heppel 1969, 1971; MacAlister 1977c; Thompson and MacLeod 1974), primarily in the periplasmic space, or at the cell surface (Cheng et al. 1970a, 1971; Cheng and Costerton 1973, 1977; Costerton 1973; Costerton and Marks 1977; Dane et al. 1965; Kushnarev and Smirnova 1966; Lindsay et al. 1973; Mac Alister et al. 1977 a, b, c; Nisonson et al. 1969). Recently Poirier and Holt (1983a) have determined that the degradative phosphatases, acid and alkaline phosphatase in Capnocytophaga appear to be located external to the cytoplasmic membrane (inner membrane), within the periplasm, as well as associated with the cell wall (outer membrane). Poirier and Holt (1983b) have presented data suggesting that localization of alkaline phosphatase activity in Capnocytophaga varies somewhat as a function of growth phase of the organism. In mid-logarithmic phase of growth, alkaline phosphatase activity appeared discretely confined to the periplasmic space whereas stationary phase cells exhibited both a periplasmic localization of the enzyme and a pronounced outer membrane localization. It is not known whether the alkaline phosphatase was released from the periplasm and then reabsorbed onto the membrane surface or was excreted through the membrane as membrane associated enzyme. The phosphatases of Capnocytophaga tend to remain cell associated during the entire growth cycle.

Sonication has been shown to be effective in releasing soluble bacterial components, as well as separating the outer and cytoplasmic membrane (DeLeig and Witholt 1977; Kulpa and Leive 1976). French pressure disruption has proved to be the most
effective method for releasing phosphatases from *Capnocytophaga* species. This method also releases soluble bacterial enzymes and separates outer and cytoplasmic membranes (Poirier and Holt 1983a).

The current studies compare the effects of employing three different methods of cell disruption on recovery of protein and enzyme activity from *Bacteroides intermedius*. It is not clear why with French pressure disruption substantial collagenolytic activity remains associated with the large cell fragments, while alkaline phosphatase and malate dehydrogenase activities are quantitatively released into the supernatant fraction. It is possible that some of the collagenolytic activity but not alkaline phosphatase or malate dehydrogenase activities is loosely membrane-associated or reassociated with membranes under the conditions employed for this cell disruption study.

Discontinuous sucrose density gradient centrifugation of low speed supernatant fractions of French pressure disrupted stationary phase *B. intermedius* and *B. gingivalis* demonstrate that extremely small amounts of collagenolytic activity appear to be associated with the outer membrane rich fraction (Figure 5 peak I) and the inner membrane rich fraction (Figure 5 peak II). However, most of the collagenolytic activity appears to be associated with the soluble fraction of the cell (Figure 5 peak III) along with alkaline phosphatase and MDH activities. This data supports a predominantly periplasmic or cytoplasmic loca-
tion of this enzyme under these experimental condition. Using the same technique, Poirer and Holt (1983a) have demonstrated outer membrane localization for *Capnocytophaga* acid and alkaline phosphatase activities. To date, no studies have appeared describing localization of *Bacteroides* species collagenolytic activity. Our data are in agreement with those of Gibbons and MacDonald (1962) who described a collagenolytic enzyme from an oral strain of *Bacteroides melaninogenicus* which was intimately associated with the cell but released during autolysis. Mayrand et al. (1980) have asserted that oral strains of *Bacteroides asaccharolyticus* possesses a cell-bound collagenase which is oxygen sensitive and requires the presence of a reducing agent in the assay buffer in order for full activity to be expressed. Our data are not in agreement with their findings. *Bacteroides gingivalis* (W) does possess a cell associated collagenase, but it does not appear that large amounts of this enzyme are bound to any bacterial components under our conditions of growth and isolation. Furthermore, addition of cysteine or dithiothreitol to the assay system had no effect on the percent collagenolysis observed.

Controlled osmotic shock, which according to Heppel (1971) functions to increase the permeability of the outer membrane (cell wall), will result in release of unbound periplasmic protein assuming that adequate outer membrane permeability is achieved. Heppel (1971) has reported release of 100% of alkaline phosphatase activity with release of only 3.5% of the total protein with osmotic shock of *E. coli*. In some organisms, osmo-
tic shock gives a poor yield of periplasmic proteins. In *S. typhimurium* for example, only 10 to 20% of the acid phosphatase was released by osmotic shock even though the enzyme was shown to be entirely periplasmic (Uerkvitz and Beck 1981; Chatterjee et al. 1976). There was no index of outer membrane permeability (i.e. protein released) reported in these studies. *Pseudomonas aeruginosa* has been osmotically shocked using 20% sucrose without EDTA and only 20-40% of alkaline phosphatase was released, the remainder was found to be attached to the cell wall (Cheng et al. 1971). Poirier and Holt (1983a) have osmotically shocked *Capnocytophaga* and have obtained only partial release of phosphatases, the remainder appear to be associated with the outer membrane. Since *Bacteroides* collagenolytic activity appears to be soluble, it should be released by osmotic shock if it is located within the periplasmic space and if adequate outer membrane permeability is achieved. It was not possible to osmotically shock stationary phase cells because the cells stuck together in large clumps and required such vigorous pipetting to resuspend them that much cell breakage ensued. Using dilute solutions of log phase *B. intermedius* (Table 5) conditions have been achieved which have resulted in a large release of collagenolytic and alkaline phosphatase activities (~50% and 38% respectively) with release of only about 3% of the total cell protein. This data supports a periplasmic localization for *Bacteroides* collagenolytic activity.

Spheroplasting is another method by which periplasmic pro-
Proteins can be released from bacteria (Poirier and Holt 1983a). Attempts to spheroplast *Bacteroides* have been unsuccessful. The observation that whole bacteria are able to carry out collagenolysis suggests that collagenolytic activity may be located at the cell surface. Since the activity does not appear to be tightly bound to outer membrane and yet may be periplasmic, it is possible that in situ collagenolytic activity is loosely associated with outer membrane and hence easily removed from this structure. In order to more definitively assess the localization of *Bacteroides* collagenolytic activity, monoclonal antibody to *Bacteroides* collagenase could be prepared and localization should be possible by immunoelectronmicroscopy. Labelling of surface proteins with $^{125}$I is a useful technique for assessing what percentage of the cell collagenase has a surface location (Bolton and Hunter 1973) and this study awaits purification of the enzyme. It would also be interesting to compare the percent of total collagenolytic activity appearing on the bacterial surface in the presence and absence of collagen substrate. It is possible that in the presence of substrate the cellular distribution of enzyme is altered.

**Binding Studies**

If collagenolysis by *Bacteroides* species is an important feature of collagen loss in periodontal disease then the collagenolytic activity must reach host collagen. Results obtained from the localization studies suggest that proximity of collagenolytic activity to collagen could occur in essentially
two ways: 1) lysing cells appear to be able to release the activity and the soluble enzyme could reach host collagen by diffusion and 2) the *Bacteroides* cell may be located proximal to host collagen. Attachment proteins may serve as "modulators" or "mediators" of interactions between bacteria and host tissue cells or components. These interactions may also function to regulate colonization of host tissue by bacteria. In addition, "linker" attachment proteins may function to maintain bacteria proximal to a nutrient source, i.e. *Bacteroides* proximal to collagen. The results of initial studies of binding of *Bacteroides* to the attachment proteins laminin and fibronectin suggests that under the same experimental conditions, a strain of *Bacteroides gingivalis* bound laminin but not fibronectin. In addition, two strains of *Bacteroides capillus* failed to bind laminin under identical conditions suggesting that laminin binding may be a unique property of some organisms.

Preliminary results suggest that binding of laminin to *Bacteroides gingivalis* (W) is irreversible since a large excess of unlabelled laminin failed to displace $^{125}\text{I}$-laminin bound to bacteria. These results are in agreement with those reported by Switalski et al. (1983) for binding of *S. pyogenes* to laminin and Speziale et al. (1984) for binding of *S. pyogenes* to fibronectin. When a large excess of unlabelled laminin was added at the same time as the labelled protein, the binding of $^{125}\text{I}$-laminin to *B. gingivalis* (W) was inhibited, indicating that unlabelled laminin can successfully compete with the iodinated protein for the
bacterial binding site. Incubation of log phase *B. gingivalis* cells with increasing amounts of $^{125}$I-laminin was accompanied by an increase in radioactivity bound to the bacteria (Figure 9). At low concentrations of added laminin, the amounts of labelled protein bound to bacteria increase rapidly, whereas above 70 ug per sample little change in the amount of laminin bound is observed. These results are compatible with a specific binding of laminin to a limited number of sites on the surface of the bacteria, however, it is also compatible with binding of laminin to bacteria that is nonspecific and that increases with the amount of radioactivity added. Competition experiments using a large excess of other proteins would allow assessment of these proteins as potential inhibitors of binding of laminin to *B. gingivalis* (W). If many other proteins inhibit the binding of laminin to *B. gingivalis* (W) this could indicate that binding of laminin to this strain is relatively unspecific. Failure of other proteins to interfere with laminin binding is compatible with presence on the bacterial surface of sites that preferentially bind laminin. Since binding of laminin to *B. gingivalis* (W) is functionally irreversible, the necessary conditions for Scatchard plot analysis are not fulfilled (Klotz 1982; Speziale et al. 1984). A Scatchard plot analysis has been included here for purposes of discussion and no conclusions should be made on the basis of this data. If all binding sites are occupied at saturation, then the number of laminin binding sites per bacterial cell is 1500 assuming a molecular weight of 900,000 for laminin
and assuming that $6.7 \times 10^8$ bacteria bind 1.5 ug laminin at saturation. This is consistent with the number of fibronectin binding sites reported to be on the surface of *S. aureus* (Proctor et al. 1982). An apparent $K_A$ of $1.3 \times 10^{10}$ can be calculated from the slope of the Scatchard plot. This $K_A$ is comparable to those calculated for binding of fibrinogen to staphylococci (Howayer et al. 1982) and binding of fibronectin to *S. pyogenes* (Speziale et al. 1984). The time dependence of the binding varied somewhat for log and stationary phase cells and in addition, it appears that stationary phase cells bind somewhat more laminin than do log phase cells. Different behavior of log and stationary phase cells was also noted in localization studies, and the fact that stationary phase cells clump in the presence of laminin and log phase cells do not, lends support to the hypothesis that log and stationary phase cells may have quite different surface composition. The biological significance of this clumping is unclear. The clumping of stationary phase cells by laminin may reflect multiple binding sites for stationary phase *B. gingivalis* (W) on the laminin molecule, and/or multiple laminin binding sites on the bacterial surface. Laminin fragments, produced by limited proteolysis, may be useful in assessing reversibility of binding of laminin to stationary phase cells.

The data obtained in the current studies suggest that it may be possible to release functional laminin binding activity from *Bacteroides gingivalis*. However, the data could also be accounted for by the presence of residual trypsin activity in the
"trypsinate" degrading $^{125}\text{I}-\text{laminin}$ used in the binding assay. This possibility could be examined by gel electrophoresis of $^{125}\text{I}-\text{laminin}$ exposed to "trypsinate", followed by autoradiography of the gel to look for laminin breakdown products. It is also possible that trypsin treatment of bacteria releases proteolytic activity capable of degrading $^{125}\text{I}-\text{laminin}$. If, however, functional laminin binding activity is released from the bacteria by trypsin treatment, then this activity should be removed from the "trypsinate" by passing this material through a laminin-sepharose column.

In order to assess the importance of attachment protein binding activities as factors influencing bacterial colonization of the periodontal pocket, it would be useful to determine whether binding and non-binding mutants of the same organism vary in their ability to establish as members of the pocket microflora in an appropriate animal model for human periodontitis. In addition to providing basic information on bacterial ecology and interactions, such knowledge may permit identification of mechanisms for selectively interfering with host colonization by pathogens.
Conclusions

In summary the following conclusions can be drawn from the data presented in this thesis:

1. All species of *Bacteroides* examined appear to possess collagenolytic activity against native collagen in solution and against collagen in reconstituted fibrils.

2. Collagenolytic activity is inhibited by serum and calcium ion depletion.

3. The collagenolytic activity appears to remain associated with the cell during all growth phases and is recoverable from the cell as a soluble enzyme.

4. Intact cells appear to be capable of carrying out collagenolysis.

5. One strain of *Bacteroides gingivalis* (strain W) appears to be able to bind the attachment protein laminin.
Table 1

Properties of Procaryotic Collagenases
<table>
<thead>
<tr>
<th>Procaryote</th>
<th>Localization of Collagenase</th>
<th>Substrate(s)</th>
<th>Bond Specificities</th>
<th>Activators</th>
<th>Inhibitors</th>
<th>Miscellaneous</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leucosidium histolyticum</strong></td>
<td>elaborated extracellularly</td>
<td>collagen (native)</td>
<td>ε-gly-ala (faster)</td>
<td>Ca**</td>
<td>EDTA</td>
<td>Im**-metalloenzyme</td>
<td>Kell, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>other proteins that possess specific sequences</td>
<td></td>
<td></td>
<td>Cysteine</td>
<td>MW 70,000</td>
<td>Harper, 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>synthetic peptide substrates</td>
<td>x-gly-pro</td>
<td></td>
<td>Isopropyl alcohol</td>
<td>Dreisbach &amp; Merkel, 1978</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>yields less active forms on partial degradation</td>
<td>Emo et al., 1981</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>probably constitutive</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pH optimum 7.4</td>
<td></td>
</tr>
<tr>
<td><strong>Vibrio alginolyticus</strong></td>
<td>elaborated extracellularly</td>
<td>collagen (native)</td>
<td>ε-gly-ala (faster)</td>
<td>histidine</td>
<td>EDTA</td>
<td>Im**-metalloenzyme</td>
<td>Kiel, 1979</td>
</tr>
<tr>
<td>(formerly Achromobacter (ophagus)</td>
<td></td>
<td>β-casein and other proteins that possess specific sequences</td>
<td></td>
<td>residue required for activity</td>
<td></td>
<td>MW 70,000</td>
<td>Harry &amp; Kell-Dieua, 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>x-gly-pro</td>
<td></td>
<td>Zn**</td>
<td>Two identical subunits</td>
<td>Kiel-Dieua &amp; Kiel, 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bromoacetone</td>
<td>only dimer is active</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>O-phenanthroline</td>
<td>yields active forms on partial degradation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>inducible</td>
<td></td>
</tr>
<tr>
<td><strong>Vibrio B-30</strong></td>
<td>elaborated extracellularly</td>
<td>collagen (native)</td>
<td>ε-gly-pro-gly-gly-pro-ala</td>
<td>Ca**</td>
<td>EDTA</td>
<td>MW 105,000 tetramer</td>
<td>Vaite et al., 1980</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>p-Chloro mercurobenzoate</td>
<td>Dreisbach &amp; Merkel, 1978</td>
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<td></td>
<td>Isopropyl alcohol</td>
<td>yields active form on partial degradation</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>pH optimum 7.6</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>inducible</td>
<td></td>
</tr>
<tr>
<td><strong>Vibrio vulnificus</strong></td>
<td>elaborated extracellularly</td>
<td>collagen (native)</td>
<td>?</td>
<td>?</td>
<td>EDTA</td>
<td>constitutive(?)</td>
<td>Smith &amp; Merkel, 1982</td>
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<tr>
<td><strong>Bacillus sp.</strong></td>
<td>elaborated extracellularly</td>
<td>collagen (native)</td>
<td>?</td>
<td>Ca**</td>
<td>EDTA</td>
<td>Metalloenzyme one enzyme</td>
<td>Loesche et al., 1974</td>
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<tr>
<td>(Cereus***)</td>
<td></td>
<td>synthetic peptide substrate</td>
<td></td>
<td>(slight activation)**</td>
<td></td>
<td>MW 350,000</td>
<td>Soderling &amp; Paumio, 1981</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cysteine</td>
<td>pl 5.18</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>pH optimum 8.0</td>
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</tr>
<tr>
<td><strong>Brucellosis melitensis</strong></td>
<td>remains cell-associated</td>
<td>collagen (native)</td>
<td>?</td>
<td>Ca**</td>
<td>EDTA</td>
<td>MgCl₂</td>
<td>Gibbons &amp; MacDonald, 1964</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cysteine</td>
<td>pH optimum 6.8 - 7.3</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Thioglycolate</td>
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</tbody>
</table>
Table 2

Collagenolytic activity of representative stationary phase *Bacteroides* species. Collagenolytic activity is expressed as the mean percent of total radioactivity solubilized over 20 hours using the standard soluble collagen assay. Three trials were performed using different batches of cells. Results from one representative trial are shown. Each mean percent and standard deviation were calculated from three replicate samples. Background activity (5.7%) has been subtracted from all values. Collagen solubilization by trypsin (50ug/ml) and *Clostridium histolyticum* collagenase A (100 ug/ml) averaged 3.3% and 57% respectively.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
<th>Collagenolytic Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell Sonic Supernatant</td>
</tr>
<tr>
<td>Bacteroides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-gingivalis (W)</td>
<td>Kornman (Conn)</td>
<td>87.9±2.4</td>
</tr>
<tr>
<td>-gingivalis (1416.10)</td>
<td>Kornman (Conn)</td>
<td>41.2±3.5</td>
</tr>
<tr>
<td>-capillus (ATCC 33690)</td>
<td>Kornman (Conn)</td>
<td>34.1±4.5</td>
</tr>
<tr>
<td>-capillus (ATCC 33691)</td>
<td>Kornman (Conn)</td>
<td>41.0±3.0</td>
</tr>
<tr>
<td>-oris (ATCC 33573)</td>
<td>ATCC</td>
<td>47.0±2.5</td>
</tr>
<tr>
<td>-thetaotaomicron (UC-2)</td>
<td>Kornman (Conn)</td>
<td>44.7±4.0</td>
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<tr>
<td>-fragilis (UC-4)</td>
<td>Kornman (Conn)</td>
<td>46.8±1.4</td>
</tr>
<tr>
<td>-mel. ss. melaninogenicus (ATCC 25845)</td>
<td>ATCC</td>
<td>34.2±2.7</td>
</tr>
<tr>
<td>-mel. ss. intermedius (794.01)</td>
<td>Kornman (Conn)</td>
<td>40.2±1.4</td>
</tr>
</tbody>
</table>

*These data are from Robertson et al. 1982
Table 3

Collagenolytic activity of sonic supernatants in the presence of human serum, and EDTA and after boiling. One part Tris-CaCl₂ buffer (1) or human serum (2) was added to three parts sonic supernatant and incubated at 37°C for 30 mins. A 100 ul aliquot of the incubation mixture was then assayed for collagenolytic activity. EDTA assays (3) were conducted in Tris buffer without CaCl₂. Omission of CaCl₂ from the Tris buffer in the absence of EDTA gave values similar to those in column (1). Heat sensitivity (4) was assessed by assay of sonic supernatants after heating in boiling water for 3 min. The standard soluble collagen assay was performed at 37°C for 20 hours. Two trials were run on different batches of cells. A representative trial is shown. These data are expressed as mean percent total radioactivity ± standard deviation solubilized over 20 hours. Each mean percent and standard deviation were calculated from three replicate samples.
<table>
<thead>
<tr>
<th></th>
<th>(1) Buffer</th>
<th>(2) Serum</th>
<th>(3) EDTA</th>
<th>(4) Boiled</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. gingivalis (W)</td>
<td>87.5±2.6</td>
<td>23.1±1.9</td>
<td>7.2±1.4</td>
<td>0.0±1.4</td>
</tr>
<tr>
<td>B. gingivalis (1416.10)</td>
<td>42.4±3.4</td>
<td>31.5±2.4</td>
<td>3.2±1.5</td>
<td>0.0±2.0</td>
</tr>
<tr>
<td>B. capillus (ATCC 33690)</td>
<td>31.2±4.0</td>
<td>10.9±1.5</td>
<td>1.0±2.0</td>
<td>0.1±1.1</td>
</tr>
<tr>
<td>B. capillus (ATCC 33691)</td>
<td>38.2±2.1</td>
<td>25.2±2.7</td>
<td>0.7±2.1</td>
<td>0.3±2.0</td>
</tr>
<tr>
<td>B. oris (ATCC 33573)</td>
<td>43.9±3.2</td>
<td>20.0±3.1</td>
<td>2.3±1.2</td>
<td>2.3±1.2</td>
</tr>
<tr>
<td>B. thetaiotaomicron (UC-2)</td>
<td>40.2±4.0</td>
<td>29.8±1.8</td>
<td>2.4±2.1</td>
<td>4.5±1.5</td>
</tr>
<tr>
<td>B. fragilis (US-4)</td>
<td>40.6±1.5</td>
<td>32.2±2.2</td>
<td>9.9±3.0</td>
<td>2.4±2.1</td>
</tr>
<tr>
<td>B. melaninogenicus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ss. melaninogenicus (ATCC 25845)</td>
<td>35.9±1.7</td>
<td>21.2±2.1</td>
<td>0.3±1.0</td>
<td>6.4±2.2</td>
</tr>
<tr>
<td>B. melaninogenicus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ss. intermedius (794.01)</td>
<td>40.2±3.1</td>
<td>16.4±1.9</td>
<td>0.5±2.1</td>
<td>2.3±1.1</td>
</tr>
</tbody>
</table>
Table 4

Comparison of methods of disruption for *Bacteroides intermedius* 794.01. After three washes with Buffer B, aliquots containing 2.0 gm wet weight of stationary phase cells in 10 ml of Buffer B, were treated as follows: cells were either incubated whole at 37°C for 30 mins. (controls), disrupted by French pressure, sonified or treated with toluene (5% v/v) as described in the Methods. Supernatant and pellet fractions (after centrifugation at 5000 x g for 20 mins.) derived from control cells and disrupted cells were compared with respect to percent total protein, collagenolytic activity, alkaline phosphatase activity, and malate dehydrogenase activity. Enzyme assays were performed as described in the Methods. Collagenolysis was assessed using the standard soluble collagen assay (37°C, 20 hours). One trial was performed using a total of 8.0 gm wet weight of cells. Protein determination and enzyme assays were run in duplicate. Data is expressed as the mean percent of total activity in a fraction. The mean and standard deviation of the difference between duplicates was:

- 5.1% ± 6.8 for proteins
- 6.3% ± 6.7 for collagenolytic activity
- 3.4% ± 4.2 for alkaline phosphatase
- 5.9% ± 6.2 for MDH
<table>
<thead>
<tr>
<th>Method</th>
<th>Protein</th>
<th>Collagenase</th>
<th>Alk. Phos.</th>
<th>M.D.H.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed, undisrupted cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.4%</td>
<td>7.0%</td>
<td>1.0%</td>
<td>3.5%</td>
</tr>
<tr>
<td>Pellet</td>
<td>99.6%</td>
<td>93.0%</td>
<td>99.0%</td>
<td>96.5%</td>
</tr>
<tr>
<td>French Pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>87.9%</td>
<td>62.4%</td>
<td>96.3%</td>
<td>94.7%</td>
</tr>
<tr>
<td>Pellet</td>
<td>12.1%</td>
<td>37.6%</td>
<td>3.7%</td>
<td>5.3%</td>
</tr>
<tr>
<td>Sonication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>73.2%</td>
<td>64.2%</td>
<td>93.7%</td>
<td>*</td>
</tr>
<tr>
<td>Pellet</td>
<td>26.8%</td>
<td>35.8%</td>
<td>6.3%</td>
<td>*</td>
</tr>
<tr>
<td>Toluene Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>11.1%</td>
<td>23.4%</td>
<td>38.6%</td>
<td>*</td>
</tr>
<tr>
<td>Pellet</td>
<td>88.9%</td>
<td>76.6%</td>
<td>61.4%</td>
<td>*</td>
</tr>
</tbody>
</table>
Osmotic shock, *Bacteroides intermedius* 794.01. 0.1 gm (wet weight) of log phase cells in 30 ml of solution (d) (see Methods) was prepared and after collection by centrifugation (5000 X g for 20 mins.) bacteria were osmotically shocked by rapid dispersal in 30 ml of cold distilled water. Two trials were performed using different batches of cells. One trial is represented here. Protein determinations and enzyme assays were performed on duplicate samples as described in the Methods. Collagenolysis was assessed using the standard soluble collagen assay (37°C, 20 hours). The data is expressed as the mean percent of total activity in a fraction. The mean and standard deviation of the difference between duplicates was:

4.3% ± 5.0 for proteins  
5.2% ± 6.1 for collagenolytic activity  
3.2% ± 3.9 for alkaline phosphatase
### TABLE 5

Percent of Total Activity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Collagenolytic Protein</th>
<th>Collagenolytic Activity</th>
<th>Alkaline Phosphatase</th>
<th>M.D.H.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose supernatant</td>
<td>7.7</td>
<td>*</td>
<td>2.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cold water wash</td>
<td>2.8</td>
<td>50.5</td>
<td>37.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cell pellet</td>
<td>89.5</td>
<td>49.5</td>
<td>60.4</td>
<td>trace**</td>
</tr>
</tbody>
</table>

*Below trypsin levels
**Too low to detect reliably
N.D. = not detected
Figure 1

SDS polyacrylamide disc gel electrophoresis of collagen - *B. gingivalis* collagenolytic reaction products. Electrophoresis was conducted by the method of Furthmayr and Timpl (1971). Gel (A) represents rat tail tendon collagen at 1 mg/ml. Chains characteristic of undigested collagen were observed. The pattern resulting from electrophoresis of *B. gingivalis* (1416.10) sonic supernatant is shown in (B). The reaction products of collagen plus *B. gingivalis* (1416.10) sonic supernatant at zero time and after incubation 37°C for 3 h prior to electrophoresis are depicted in gels (C) and (D), respectively.
Figure 2

Time dependence of collagenolysis by the sonic supernatant of stationary phase *Bacteroides gingivalis* (W). Sonic supernatant was prepared using 1.0 gm (wet weight) of stationary phase bacteria suspended in 10 ml of Buffer A, as described in the methods. Each assay (soluble) was performed using 100 ul of sonic supernatant. Three trials were run on different batches of cells. One representative trial is shown. Data is represented as mean percent collagen solubilized from duplicate samples. Blanks were subtracted from samples for each time point. The mean percent difference between duplicate samples was 1.22 with a standard deviation of 1.19.
% Collagen Solubilized

Time (hours)
Figure 3

Collagenolysis as a function of enzyme concentration for purified *Clostridium histolyticum* collagenase (included for purpose of comparison), and sonic supernatants prepared from *Bacteroides gingivalis* 1416.10 and *Bacteroides gingivalis* (W). Standard soluble collagen assay as described in the Methods was employed and the reactions were run for 20 hours. Three trials were performed on different batches of cells. One representative trial is shown. Blanks were subtracted from each sample value. The means and standard deviation of the difference between duplicate samples were:

46.0 cpm ± 32.3 for *B. gingivalis* 1416.10
64.5 cpm ± 57.2 for *C. histolyticum*
106.8 cpm ± 140.0 for *B. gingivalis* (W)
Collagenolysis by sonic extract, sonic supernatant and sonic pellets as a function of length of time of sonication for *Bacteroides gingivalis* 1416.10. Standard soluble collagenase assay was performed as described in the Methods (37°C, 20 hours). Cells were sonified for 3, 6, 9, 12, 15 or 21 mins. Two trials were performed on different batches of cells and the results of one trial are shown. The data are presented as mean cpm of duplicate samples. The mean and standard deviation of the difference between the duplicates was 155.0 cpm ± 120.1.
Collagen Solubilized (cpm x 10^{-3})

SONIC EXTRACT

SONIC SUPERNATANT

SONIC PELLET

Total Time of Sonication (min)

O 0 3 6 9 12 15 18 21

-76-
Figure 5

Fractionated density gradient following sedimentation velocity centrifugation at 100,000 X g for 3 hours of French press disrupted low speed supernatant fraction prepared from Bacteroides intermedius 794.01. A total of six such gradients were prepared from three different batches of cells. The results shown are derived from one representative fractionated gradient. Fractions (1.0 ml) were collected from the bottom of the tube. The discontinuous sucrose gradient is shown in the insert on the upper left. Protein determinations and enzyme assays were performed in duplicate. Collagenolysis was assessed using the standard soluble collagen assay (37°C, 20 hours). Data plotted is the mean of duplicate samples.
Figure 6

Electron micrograph of Peak I, Figure 5, the lower band on the gradient. The cell wall (cw) and associated peptidoglycan (pg) can be seen. Total magnification: 20,000 x.
Figure 7

Electron micrograph of Peak II, Figure 5, the upper band on the gradient. Note single membrane bound vesicles (smv). Total magnification: 15,000 x.
Figure 8

Time dependence of binding of $^{125}$I-laminin to log and stationary phase *Bacteroides gingivalis* (W). Incubations were performed at room temperature. Three trials were run using six different batches of cells. One representative trial is shown for log and stationary phase cells. The mean percent difference of duplicates for log phase cells was 0.41% with a standard deviation of 0.33, for stationary phase cells 0.52% with a standard deviation of 0.43.
% Laminin Bound/10^9 Cells

Time (hours)

- Stat Phase
- Log Phase
Saturation curve: Binding of $^{125}$I-laminin to log phase Bacteroides gingivalis (W). $6.8 \times 10^8$ bacteria were incubated with increasing concentrations of $^{125}$I-laminin (specific activity $1.8 \times 10^4$ cpm/ug) for one hour at room temperature. Four trials were conducted, each employing a different cell concentration ranging from $10^8$ to $10^9$ cells/sample. One representative trial is shown. Duplicate points are plotted for each laminin concentration shown. Scatchard plot of data in Fig. 9 is shown in the insert.
Laminin Bound (ug)

Laminin Added (ug)

B/F \times 10^2
Figure 10

Inhibition of binding $^{125}$I-laminin to stationary phase Bacteroides gingivalis (W) by trypsin-released material from Bacteroides gingivalis (W). Incubations were performed at room temperature. Three trials were performed on different batches of cells. One representative trial is shown. The mean percent difference of duplicate samples was 5.7% with a standard deviation of 5.8.
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