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Characterization of Collagenase Production by Subspecies of Black Pigment Producing Bacteroides

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CHARACTERIZATION OF COLLAGENASE PRODUCTION BY

SUBSPECIES

of

Black Pigment Producing

Bacteroides

by

ANTHONY BUDZINSKI D.D.S.

A Thesis Submitted to the Faculty of the Graduate School

of Loyola University of Chicago in Partial Fulfillment

of the Requirements for the Degree of

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LIFE

Anthony John Budzinski was born to Lucius and Fernadine Budzinski in Chicago, Illinois on July 25, 1956. He graduated from Saint Patrick High School in June of 1974 and received his Bachelor of Science degree from the Department of Biology at the University of Illinois, Urbana, in June of 1978. He began his Masters of Science research in the Department of Oral Biology at Loyola University School of Dentistry, Maywood, under the guidance of Dr. James C. Hagen in August of 1978. In 1980 he received second place at the American Association of Dental Research Young researchers competition for his abstract; "Characterization of Collagenase Production by Oral Subspecies of Bacteroides melaninogenicus." In September of 1980 he began his D.D.S. studies at Loyola University School of Dentistry, Maywood. He received his D.D.S. degree with dental honors in May of 1984. He is a member of Psi Omega, the Chicago Dental Society and the American Student Dental Association.

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CHAPTER 1

INTRODUCTION

In the past, the most widely recognized and best described anaerobic infections were due to microorganisms acquired exogenously. A classic example of this type of infection is clostridial myonecrosis or "gas gangrene", caused by microorganisms of the genus _Clostridium_. Infections usually develop following trauma and involve contamination of wounds by Clostridial spores which are widely distributed in nature (67). At the site of injury, the necrotic tissue, blood clots and foreign bodies greatly reduce blood supply to the wound. The anaerobic conditions necessary to germinate spores and initiate the infection (19) are thereby created. The main pathogenic quality associated with these clostridia is their capability of elaborating large quantities of destructive enzymes, such as alpha toxin. Alpha toxin possesses phospholipase C or lecithinase activity. In addition Clostridium possess collagenase and neuramidase (34), producing infection by causing destruction of both tissue and blood cells (13).

The pathogenic qualities possessed by these organisms, along with their wide distribution in nature, have made them extremely dangerous to man. Improvement of general management of these diseases along with the discovery of effective antimicrobial agents have enabled both the physician and

dentist to control the occurrance of these primary infections in man. Unfortunately, as control of these exogenously acquired anaerobic pathogens became possible, a more complex and less understood class of endogenously acquired anaerobic infections has occurred to take their place.

Obligately anaerobic microorganisms are found endogenously as predominant residents of the skin and mucous membranes (82,91). Although anaerobes are normally found on, or in the body, they may become pathogenic when introduced outside of their normal environment. In fact it has been shown that the majority of anaerobic infections arise from the normal microflora of the body and could be called opportunistic in nature (94,33,2,8,40). Under certain predisposing circumstances, including surgery, malignencies (11), prior antibiotic therapy, diabetes and increased longevity (16), anaerobes from the endogenous microflora have an opportunity to penetrate the normally protective mucosal barrier and begin the infectious process. These infections are caused by the normal microflora which reside at or around the site of infection and are generally polymicrobic with a mixed flora of both obligately and facultatively anaerobic organisms (17-21,62). Infections commonly involves indigenous anaerobes and include brain abscess (37), lung abscess (4,105,106), appendicitis with peritonitis (96), vulvovaginal abscess (22), and oral infections (52,109,55,56,38). The anaerobes most commonly isolated from such infections include Fusobacteria, Bacteroides melaninogenicus, Peptostreptococcus,

peptococcus and Bacteroides fragilis (2). These nonsporulating anaerobic bacteria are rarely isolated alone, and are usually associated with various other anaerobic and aerobic organisms which exist together in a synergistic relationship (75,86). The fact that each organism must produce the disease in combination when they can not do so alone suggests that their combination is of mutual benefit while it is harmful to the common host. Mixed infections are extremely complex and must be studied in terms of each individual organisms' contribution to the whole as well as the effect of these organisms synergistic relationship on the host (71).

Recent publications from the medical and dental literature have documented an increased recovery rate of the anaerobic microorganism Bacteroides from clinical specimens, and have implicated the black pigment-producing oral Bacteroides (BPPOB) as playing a major role in the pathogenesis of periodontic and endodontic infections (44,57,79,100,101,113,122,124). While much work has been performed demonstrating the pathogenicity of these organisms in medical infections, there is a paucity of information concerning their actual role in dentally related disease.

The BPPOB (B. asaccharolyticus, B. menlaninogenicus, subsp. intermedius and subsp. melanininogenicus) are thought to be an essential pathogenic component of inflammatory periodontal disease (59,60,109). Due to the prevalence of B. asaccharolyticus in periodontal isolates and the fact that there appears to be a direct correlation between level of

this organism and severity of infection, it has been suggested that B. asaccharolyticus is indicative of, and pathologically involved with, periodontitis (71,101,113). The intricacy of these mixed, polymicrobic anaerobic infections has made their study tedious and complex, resulting in a paucity of knowledge concerning their etiology and pathogenesis (58).

One of the most prevalent and poorly understood types of polymicrobic mixed anaerobic infections is Inflammatory Periodontal Disease (IPD) (16,59). Human IPD can be observed as at least three clinically distinct but pathogically interrelated types; acute ulcerative gingivitis, periodontosis and periodontitis. Although all three types of IPD remain clinically distinct, two findings remain consistent. First is the mandatory involvement of the oral microbial flora (41,110,59,16,42,53,100,101,102,118), and second is the destruction of gingival collagen fibers, the major supporting tissue of the periodontium.

First, a direct association can be made between the oral microbial mass and the incidence of IPD (50). The microflora associated with periodontal disease is extremely complex and in many ways reflects the flora of the normal oral cavity (38,16,59,58,10). Bacteria found in the disease state include large numbers of various spirochetes, vibrios, Bacteroides species, anaerobic streptococci, anaerobic diptheroids, Fusobacteria, Capnocytophaga, Actinobacillus, and other organisms (93,55). Although the specific microbes involved in each clinical type of IPD may differ, there often

is a dramatic increase in numbers of microorganisms isolated from the disease state. This is an important observation, since the mixed microbiota isolated from IPD possess certain pathogenic qualities, as exemplified by infections attributable to human bites (74,28), typical ulcers, noma, and like conditions.

Perhaps more importantly, shifts in flora composition are observed in IPD. There is a shift from the predominant gram-positive facultative flora supragingivally and in normal pockets to the predominantly anaerobic gram-negative rod flora of the deep periodontal pocket (30). The gram-negative rod most commonly isolated from periodontitis, and implicated as a causative agent of this disease is Bacteroides melaninogenicus.

This bacillus has three separate and distinct subspecies. B. melaninogenicus subspecies melaninogenicus intermedius and asaccharolyticus. Each subspecies differs both biochemically and physiologically. It was determined that B. melaninogenicus subsp. asaccharolyticus was sufficiently different from the other subspecies to be regarded as a separate species designated Bacteroides asaccharolyticus.

BPPOB are strictly anaerobic, gram-negative bacillae. It is commonly found as normal flora in the gastrointestinal tract, vagina (33) and oral cavity (108). In the oral cavity, BPPOB are found in concentrations approximating 10^6 organisms per gram of gingival debris (18,45). This rod increases in

number in plaque, with age (5), gingivitis (104) and chronic periodontitis (103,47,15).

Experimental evidence has shown <u>B. melaninogenicus</u> to be required for the pathogenicity of oral flora associated with periodontal disease (59,69,107). As with other infections involving <u>Bacteroides</u> the bacterial etiology of periodontal disease involves a polymicrobic mixed flora (111). Oral flora from gingival debris has been shown to cause infections in guinea pigs and rats (109). It was demonstrated that when <u>B. melaninogenicus</u> was eliminated from a naturally occurring mixture of human oral flora, such infections could not be induced. Additions of <u>B. melanino-genicus</u> back to the inoculum completely restored infectivity (71). MacDonald et al (56) demonstrated that this complex bacterial mixture could be reduced to four organisms and still retain infectivity (59). These include <u>B. melanino-genicus</u> two other <u>Bacteroides</u> sp., and a facultative diphtheroid.

It has been demonstrated that human oral gram-negative microflora posssess endotoxin typical of endotoxin found elsewhere in nature (76) and that these endotoxins could induce inflammatory lesions in the vagina (76), the mucous membranes (88,89) and the gingiva (89). The BPPOB have been shown to possess a physiologically active endotoxin (76). Endotoxin levels were also found to be increased in the affected gingival tissue (14), and even in the cementum of teeth. Although a causative relationship was not established, there was a statistically significant correlation between

amount of endotoxin in human gingival exudate during perio-
dontal disease, and clinical degree of inflammation
(including tissue destruction).

Further evidence implicating B. melaninogenicus as
an essential pathogenic components of inflammatory
periodontal disease is its ability to produce an arrray of
substances potentially damaging to the periodontium. These
substances include collagenase, fibrinolysin, deoxyribo-
nuclease, chondroiten sulfatase, gleatinase, lipase, hydrogen
sulfide, indole, ammonia, organic acids and other cytotoxic
substances (25,32,59,60,70,83,95,115,122,112,118,119,127).
In spite of the fact that B. melaninogenicus has the
ability to produce this wide array of potentially destructive
substances, and that these toxic factors can cause cell death
(12), the actual role of B. melaninogenicus in the patho-
genesis of such mixed infections as human periodontal disease
is unclear. It is not likely that this organism alone
initiates the disease process (71). However, once the
disease is initiated it is likely that products of B.
melaninogenicus and other organisms (115) play a role in
continuing progression and severity of the disease.

The second consistent finding in IPD is a destruction
of collagen fibers. Collagen is the most prevalent protein in
man. It is the major consitituent of teeth and the supportive
tissue which surrounds them. The collagen molecule consists
of three helical polypeptide chains which are hydrogen bonded
to each other forming a triple helical tropocollagen macro-

molecule. These tropocollagen macromolecules then aggregate in one-quarter staggered arrays to form the collagen fibril.

Because of the unusual triple helical structure of collagen, very few enzymes are able to attack it. The only known enzyme able to digest native collagen is collagenase (114). By definition, collagenase are enzymes which are capable of digesting native collagen fibrils under physio-logical conditions of pH and temperature, and which act by cleaving the helical part of the molecule . This definition excludes proteolytic enzymes such as trypsin and pronase which might attack the the non-helical protions of the molecule.

The degradation of collagen by collagenase has been implicated as one of the major forces of gingival destruction in inflammatory periodontal disease. In fact, the degradation of collagen has been shown histochemically to be the earliest observeable lesion in IPD (24). Although the exact mechanisms of collagen loss have not been established, two major sources of collagenolytic enzymes exist. First, as can be seen in physiologic remodeling during development, the maintenance of tissue morphology, repair during wound healing, and destruction of collagen associated with "connective tissue disease", collagen in the oral cavity is constantly being destroyed by the body itself (91). The turnover of collagen appears to be controlled by vertebrate tissue collagenase.

The mechanisms of host tissue collagenase on the native collagen molecule is unique. All tissue collagenases cleave the native tropocollagen molecule into two large fragments. The largest of these two fragments include three quarters of the tropocollagen molecule and contains the amino terminal end of these chains. The other fragment which is a quarter portion of the tropocollagen molecule contains the original three carboxy termini.

Most interestingly, it has been suggested that the phagocytic cells involved in inflammation may be an important source of the collagen degrading collagenases (91). In support of this suggestion, Schroeder et al. (99) and Flieder et al. (27) have noted major collagen breakdown in the area of the inflammatory cell infiltrate. Periodontal disease can be described as both an acute and chronic inflammatory pathosis of the periodontium (78). The inflammatory response is activated by bacterial plaque which accumulates at the junction between teeth and gingiva, and promotes a complex series of inflammatory reactions which gradually lead to the destruction of periodontal collagen (50,78). The cellular response consists primarily of polymorphonuclear leukocytes (PMNL) and macrophage infiltration of the gingival tissue. Interestingly, PMNL and macrophages have been shown to be capable of producing collagenase in vitro (91,31).

Both active and latent forms of cellular collagenase have been found in gingival crevicular fluid in both healthy subjects and in subjects with periodontal disease (23,36, 26,92). Most of the collageanse in fluid from healthy

gingiva exists in a latent form. In contrast, fluid from inflammed gingiva contains much collagenase in active form. It has been shown that bacterial plaque is able to activate latent forms of collagenase in gingival fluid of human PMNL (90). It is postulated that bacterial plaque can cause the specific release of collagenase during phagocytosis of plaque substances and non-specific release when leukocytes are destroyed by bacterial toxins.

Bacterial endotoxin from Escherichia coli has been shown to stimulate the production of collagenase from macrophages (77,123). Futhermore it was found that the lipid moiety was the portion of the lipopolysaccharide responsible for inducing the collagenase. The biochemical nature of the factors in plaque that activate collagenase remains to be characterized. These factors may vary both quantitatively and qualitatively according to the microbial constituents of plaque.

The second source of enzyme capable of degrading native collagen under physiological conditions is from the oral microbial mass itself. The reported collagenolytic activity of BPPOB and the association of these organisms with clinical disease strongly implicates Bacteroides as a major etiologic agent of oral disease. Bacterial collagenase was first discovered in 1922 by H. Henry (39) who observed that culutral filtrates from Cl. perfringens were able to to disintegrate fresh muscle tissue, although Henry did not realize that this reaction was potentiated by bacterial

collagenase. In a similar fashion, Weinberg and Randin
(120,121) discovered that filtrates from cultures of the
anaerobe Cl. histolyticum could slowly digest small
pieces of Achilles tendon.

The implications of this finding were ignored or
forgotten until 1937 when the German biochemist Maschman (63)
found a new enzymatic ability to break down collagen by the
organism Cl. perfringens. The enzymes' existence was
later confirmed by Oakley et al. in 1946 (84) and clearly
identified as collagenase by Bidwell and Van Heyningen in
1948 (9). Many aerobic and anaerobic microorganisms were
tested for their ability to produce a collagenase, although
Clostridiuim was the only one found to produce the enzyme
(66) the genus Bacteroides was not tested. In 1961, Gibbons
and MacDonald were the first investigators to describe the
degradation of collagenous substrates by Bacteroides (29).
Other investigators then delineated conditions under which
the collagenolytic activity was demonstrated (35). Their
results suggested that collagenase was a preformed enzyme and
did not require the presence of live bacteria. In addition,
the pH of the collagen gel did not go below 6.4, consistant
with an enzyme capable of hydrolyzing native, insoluble
collagen. Robertson et al. found that enzyme activity was
enhanced when the microorganisms were grown in peptide-
depleted medium (91). It is now known that although
Bacteroides collagenase is excreted into the culture
supernatant and can be measured in this manner, it is
actually cell-associated (91,69) and results obtained by M.

Lantz, R.B. Robertson and S.C. Idolt (1983) J. Dent. Res.
62:289 Abst. No. 1076 indicate that the enzyme is contained
within the periplasmic space.

Early studies were not able to differentiate between
the different subspecies of <u>Bacteroides</u>. Even today with
the distinctions made between the species and subspecies of
black-pigmenting oral <u>Bacteroides</u>, there is much
controversy as to which of them are able to exhibit
collagenolytic activity (91,69,117). Mayrand et al. (71)
showed that oral strains of <u>B. melaninogenicus</u> and <u>B.
intermedius</u> did not exhibit any collagenase activity.
Other workers (Steffen and Hentges 1981, (115) Robertson et
al. (1982) (91) using different protocols, assay procedures,
and strains found <u>B. assachorolyticus</u> as well as other
<u>Bacteroides</u> species to be collagenolytic. Most recently
Mayrand et al. (70) using an assay described by Steffen and
Hentges (115) found that only one organism, <u>B. gingivalis</u>
formly <u>B. asaccharolyticus</u> was able to degrade collagen
under their specific set of procedures. In fact, there is
little agreement as to which of these microorganisms are
actually involved in the pathogenesis of periodontitis.

CHAPTER 2

MATERIALS AND METHODS

I. Isolation and Maintenance of Cultures

Bacteroides melaninogenicus subspecies melanino-
genicus strain 15930, B. melaninogenicus subsp. inter-
medius strain 581 and B. asaccharolyticus strain 381
were used throughout this study. Other organisms used
were B. melaninogenicus subsp. intermedius strains
strains 581B, 587, 379 and B. asaccharolyticus strains
382 and AB-1. Strains 15930 and 25261 were obtained from
the American Type Culture Collection (Rockville, Maryland).
Strains 381, 382, 379, 581, and 581B were kindly supplied
from stock cultures by the Virginia Polytechnic Institute or
from deep periodontal pockets by Dr. D.C. Birdsell of the
University of Florida (81). Strain AB-1 was isolated from
a deep periodontal pocket during a periodontal surgery at
Loyola University School of Dentistry. The oral strains of
Bacteroides listed above are referred to as black-pigment
producing oral Bacteroides (BPPOB). The identity of all
Bacteroides used in this study was confirmed by use of the
API 20A system (Analytab Products, Plainview New York), which
employed a variety of biochemical tests.

Stock cultures of each strain were stored in 1.0 ml defibrinated sheep blood (Ovine Laboratories, Chicago) at -60°C (Model SZB-659, Revco Freezer Co.). For routine work, cultures were maintained at 37°C in enriched Todd-Hewitt Broth or on blood agar (BBL) as described below.

II. Media

A. Liquid media. Several different complex media were used in these studies. Enriched Todd Hewitt Broth (enrTHB), Enriched Trypticase Soy Broth (enrTSB) and Enriched Brain-Heart Infusion Broth (enrBHI) were prepared by adding 0.5% yeast extract (BBL), 0.05% hemin (Eastman Organic Chemical Div., Eastman Kodak Co.), and 0.0005% menadione (Sigma Chem. Co.) to an appropriate amount of THB (BBL), TSB (BBL) or BHI (BBL). All liquid media used in all experiments were allowed to stand at room conditions for 48 hrs. before their inoculation.

B. Solid media. For the routine growth and transfer of BPPOB enriched Todd-Hewitt Blood Agar (enrTHBA) was prepared by the addition on 1.5% agar (Difco labs) and 5% defibrinated sheep blood (Ovine Laboratories) to enrTHB.

C. Selective media. To selectively isolate BPPOB from a variety of microorganisms found in deep periodontal pockets, media was prepared by adding 75 mg/ml kanamycin (51) (Sigma Chem. Co.) and 75 mg/ml L-Cysteine (Nutritional Biochemical Corp.) to enrTHBA. Kanamycin at this concentration will inhibit growth of most other organisms while allowing BPPOB to be isolated.

III. Anaerobic Systems

Routinely, cultures for anaerobic growth were placed in anaerobic jars (BBL) containing a platinum-palladium catalyst. Anaerobic conditions were created by flushing the jar at least three times with a mixture of 5% hydrogen, 10% carbon dioxide and balanced nitrogen (anaerobe grade, Benster Speciality Gas Co.) (43). A methylene blue indicator (BBL) was placed in all jars to monitor anaerobic conditions.

IV. Growth Determinations

In order to determine the characteristics of growth of BPPOB, B. melaninogenicus strains 15930, 25261, 581 and 381 were grown in enrTHB under anaerobic conditions at 37°C until visible turbidity could be seen. Each culture was then diluted into an appropriate number of two-day-old tubes of enrTHB to a concentration of 10^5 cells/ml and then placed in anaerobe jars under anaerobic conditions at 37°C. One tube was removed from anaerobic conditions at 0 time, and then another tube every two to four hours depending on the growth rate of the microorganisms. Serial ten-fold dilution were made from this tube into sterile saline (8.75% NaCl). A 0.1 ml aliquot was spread with a sterile bent glass rod on the surface of duplicate enrTHBA plates giving final dilutions of 10^1 thru 10^3. The plates were then placed under anaerobic conditions at 37°C

for two to four days, removed from incubation, plates containing 30-300 colonies counted, and data recorded.

A. <u>Dry weight</u>. To determine the dry weight of cultures grown in broth, duplicate tubes were inoculated. At appropriate time periods, each set of tubes was centrifuged in a IEC HN-SII centrifuge (International Equipment Co., Mass.) at 800 x g for twenty minutes to separate the cellular pellet from the supernatant. The supernatant was discarded and the pellet washed once in saline. These washed cells were centrifuged again and the supernatant discarded. The washed pellet was placed into tubes of saline and resuspended by vortexing (Scientific Industries Inc. New York). The amount of saline added was equal to the original volume of the broth cultures.

Foil laminated bake cups (Maryland Cup Corp, Maryland) were used as weighing cups. These cups were preheated in a 70°C oven (National Appliance Corp, Oregon) for two hours and then pre-weighed on a Mettler H10 balance (Mettler Instrument Corp. New Jersey). Within five minutes of preheating, 1.0 ml of the resuspended cells was added to the preweighed cups. A control cup was prepared by adding 1.0 ml of saline to a preweighed cup. These cups were then placed at 70°C overnight, removed from incubation and immediately weighed. Dry weight of cells in mg/ml was determined by subtracting the pre-weighed value of the control cup from the weight of the sample cup.

V. Azocoll Assay

A. Azocoll. Azocoll (Calbiochem., San Diego, CA) is a reddish-purple, insoluble powder prepared by dying hide powder with an azo dye made from tetrazotized binzidine and R-salt (sodium 2-naphthol-3:6 disulphonate). Azocoll is disintegrated when incubated with collagenase,(98) the action of the enzyme being indicated by liberation of a soluble red dye from the insoluble azocoll particles. The amount of color may then be measured spectrophotometrically and amount of enzyme determined by comparing to a standard curve.

B. Amount of azocoll used in assay. In order to determine the proper amount of azocoll which would detect the upper limit of the BPPOB collagenolytic activity, 2 mg, 4 mg, 8 mg, and 18 mg of azocoll were each added to five tubes containing 9.8 ml of enrTHB. A Stock collagenase solution was prepared by adding 5 mg of Clostridiopeptidase A (Sigma Chemical Co.) to 1.0 ml of distilled water and performing serial two-fold dilutions to a final concentration of 0.3 mg/ml. Finally, 0.2 ml of each dilution was added to one of each of the tubes containing the various amounts of azocoll in enrTHB. The tubes were then placed at 37°C and allowed to stand for four hours. At the end of four hours, excess of Azocoll was removed by millipore filtration (Millipore Corp., Mass) through a #1 Whatman filter paper (Whatman Limited, England). The intensity of red dye liberated was determined by use of a Klett-Sommerson Photoelectric Colorimeter (Model 800-3,

Klett Mfg. Co., Inc., New York) with a #54 filter having an appropriate spectral range of 500-570nm. It was found that all levels of enzyme completely degraded 2 mg, 4 mg, and 8 mg samples of Azocoll. However, at concentrations of 18 mg of Azocoll there was an increase in the optical density with increasing concentrations of collagenase, throughout the enzyme range indicating that there was a sufficient amount of Azocoll to be used in our experiments.

C. <u>Standard curve</u>. After the appropriate amount of azocoll to be used has been determined a standard curve of collagenase vs. azocoll was prepared using dilutions of Clostridiopeptidase A as described earlier. Enzyme dilutions ranged from 5 mg/ml to 0.3 mg/ml. Then, 0.2 ml of each dilution was added to 9.8 ml of enrTHB giving final concentrations of enzyme from 0.1 mg/ml to 0.006 mg/ml. Azocoll (18 mg) was then added to each tube and all tubes were incubated at 37°C for four hours. At the end of four hours, excess azocoll was removed by millipore filtration and intensity of red dye liberated was determined photo-metrically as described above. The optical density readings were then compared to the amount of collagenase present and a standard curve of optical density vs. collagenase concentration made. One unit of activity was defined as the amount of Clostridiopeptidase A necessaráy to give a Klett reading of 40 after a 4 hour incubation at 37°C in 18 mg of Azocoll. In further experiments this standard curve was used to determine the amount of collagenolytic activity present in BPPOB in units.

D. _The assay._   To determine the collagenolytic
activity of BPPOB, strains were grown in enrTHB under
anaerobic conditions at 37°C for two to four days. Cultures
were removed from incubation and centrifuged at 800 x g for
twenty minutes to separate supernatant from cells. The
supernatant was decanted and filtered through a 0.45
millipore filter in a millipore filter unit (Millipore
Corporation, Mass) to remove any remaining cells. The cell
pellet was washed in TES buffer (0.5M A-tris (hydro-
xymethyl) methyl 2-aminoethane and 0.05 M CaCl, pH 7.4,
Sigma Chem. Co., 3) centrifuged, and the washed pellet
suspended in an amount of TES buffer equalling the original
volume of the culture. Azocoll (0.018g) was then added to
the filtered supernatant and to the washed, resuspended
cell pellet. These tubes were then incubated at 37°C for
four hours. At the end of four hours, the remaining
azocoll was filtered out of both supernatant and cell
suspension with a #1 Whatman filter paper. The cell
suspension was further filtered with a millipore filter
unit containing a 0.45 millipore filter to remove all
cells. Color liberated from the insoluble azocoll was read
with a Klett-Sommerson Photoelectric Colorimeter and
compared to the standard curve to give quantitative amount
of collagenolytic activity exhibited by each organism.

VI. _Determination of Collagenolytic Activity_

In order to determine the collagenolytic activity of
the BPPOB, strains 581B, 587, 379, and 581, strains 381,

382, and AB-1 and strain 15930 were grown in tubes of
enrTHB under anaerobic conditions at 37°C for nine days
(29). Collagenolytic activity was determined using the
azocoll assay as described above. In this preliminary
screening, only the supernatant was tested for collagen-
olytic activity (29).

VII. Comparison of Collagenolytic Activity to Bacterial
Growth Patterns of B. melaninogenicus Subspecies

    A. Growth determinations. BPPOB strains 581, 381, and
15930 were grown in enrTHB under anaerobic conditions at
37°C for 24 hours. Each culture was then diluted into an
appropriate number of duplicate tubes of two day old enrTHB
to a concentration of 1.0×10^5 cells per milliliter.
Depending on the growth rate of the organism, both of the
duplicate tubes were removed from their anaerobic
conditions zero and every two to four hours for up to four
days. One of these duplicate tubes was used to determine
growth patterns by dry weight of organisms as described
above.

    B. Collagenolytic activity. The other duplicate tube
from above, was used to determine the collagenolytic
activity of these organisms during their growth.
Collagenolytic activity was determined using the azocoll
assay described above. Collagenolytic activity was
expressed as units per milligram dry weight of organisms.

VIII. Determination of Optimal Conditions for the

Collagenolytic Activity of B. melaninogenicus Subspecies

A. Media. B. melaninogenicus subspecies melaningenicus strain 15930, subspeices intermedius strain 581 and B. asaccharolyticus strain 381 were grown in tubes of enrTHB, enrTSB, enrBHI, and Nutrient Broth (NB) under anaerobic conditions at 37°C for nine days (29). At the end of nine days tubes were removed from incubation, and collagenolytic activity of the three organisms determined using the azocoll assay as described above. Collagenolytic activity was expressed as units per milligram dry weight of organisms.

B. Additives. In order to determine the effect of various additives on the collagenolytic activity of BPPOB, the media described above was supplimented with one of the following; 0.5% yeast extract, 0.05% hemin and 0.0005% menadione or combination of these.

IX. The Effect of Various Parameters on the Collagenolytic Activity of BPPOB

A. pH. In order to determine the effect of pH on the collagenolytic activity of the BPPOB one strain of each was grown in buffered enrTHB. Buffered enrTHB was prepared with McIlvaine buffer solution, (citrate-phosphate buffer) in place of water (72,73). B. melaninogenicus subsp. melaninogenicus strain 15930, B. melaninogenicus subsp. intermedius strain 581 and B. asaccharolyticus strain 381 were grown in 500 ml flasks of buffered enrTHB

buffered to pH 7.2 with a digital ionanalyzer (model 601A, Orion Research, Inc. Massachusetts).

Cultures were grown under anaerobic conditions at 37°C for nine days (29). At the end of nine days cultures were removed from incubation and pH was again determined and then adjusted to pH 7.2. At the same time, collagenolytic activity was determined using the azocoll assay as described above. Collagenolytic activity was expressed as units per milligram dry weight of organisms.

B. <u>Parotid gland secretion (PGS)</u>. PGS was obtained through direct cannulation using a Carlson-Crittendon cap placed directly over the Stenson's Duct and sterilized by filtration using a 0.45 millipore filter unit. The effect of PGS on the collagenolytic activity of <u>B.</u> <u>melaninogenicus</u> subsp. <u>melaninogenicus</u> strain 15930, <u>B.</u> <u>melaninogenicus</u> subsp. <u>intermedius</u> strain 581 and <u>B.</u> <u>asaccharolyticus</u> strain 381 were determined. All organisms were inoculated to enrTHB containing 1.0, 5.0 or 10.0% sterile PGS. Duplicate tubes containing 1.0, 5.0 and 10.0% sterile water were also prepared to be used as controls. All tubes were placed under anaerobic conditions at 37°C for nine days (29). At the end of nine days tubes were removed from incubation and collagenolytic activity was determined using the azocoll assay as described above. Collagenolytic activity was expressed in units per milligram dry weight of organism.

X. Electron Microscopy

A. Prefixation. Microorganisms to be visualized were removed from the surface of an enrTHBA plate and placed in 9.0 ml of modified veronal buffer, MVB, pH 6.1 (47). The tube was centrifuged at 800 x g for twenty minutes. Prefixation was completed by placing the cells in a 4% solution of gluteraldehyde in (MVB) with addition of 700 parts per million of the polysaccharide stain, ruthenium red (Sigma Chemical Co., MO) and thoroughly mixed (47). The tube was then centrifuged at 800 x g for twenty minutes and the supernatant discarded.

B. Fixation. Fixation was accomplished by placing the pellet in an OsO_4 solution (MVB, OsO_4, ruthenium red) throughly mixing the solution and cells, and letting stand overnight.

C. Dehydration. The tube was again centrifuged at 800 x g for twenty minutes and the supernatant discarded. The specimen was dehydrated sequentially in 70, 90, 95 and three 100% acetone solutions (Mallinkrodt Chemical Co., Kentucky, acetone diluted in distilled water) for one half hour each. The specimen was then centrifuged after each succesive dehydration at 800 x g for twenty minutes and the supernatant discarded.

D. Embedding. The specimen was imbedded by placing the cell pellet in 50:50 mixture of Spurr's (Polyscience, Inc. Penn.) and acetone and throughly mixed. After one hour the tube was centrifuged at 800 x g for twenty minutes and the supernatant discarded. This procedure was then

duplicated with 100% Spurr's at room temperature for twenty-four hours. Embedding was completed by transferring an appropriate amount of the stained specimen to freshly prepared 100% Spurr's in a BEEM capsule (Polyscience, Inc. Penn.) at 70°C for twenty-four to forty-eight hours.

RESULTS

I. Preliminary Findings

A. Optimal growth media

Various enriched complex media are routinely employed for the growth of anaerobic microorganisms. These media include, enrTHB, enrTSB, enrBHI, and enrNB, and were used because of their practicality, cost and, most importantly, their effectiveness for the growth of fastidious anaerobes. In order to determine a medium in which BPPOB would display their greatest collagenolytic activity, B. asaccharolyticus and B. melaninogenicus subsp. were grown in each media and the supernatant of each tested as described in Materials and Methods. As seen in Table 1, a representative of each subspecies is shown. Greatest collagenolytic activity was grown in enrTHB. Other strains tested gave similar results. Somewhat lower levels of activity could be seen in the enrTSB, while the lowest levels of collagenolytic activity were detected in the enrBHI. Both B. asaccharolyticus and B. melaninogenicus subspecies were unable to grow in enrNB. As determined by dry weight, BPPOB grew in enrTSB, with slightly less growth being observed in both the enrTHB and enrBHI media. (Data not

TABLE 1

Collagenolytic Activity[a] of <u>Bacteroides melaninogenicus</u>
Subspecies After 9d Growth[b] in Various Media

Enriched Growth Media	Collagenolytic Activity (units/mg dry weight) Subspecies/Strain		
	<u>asaccharolyticus</u> 381	<u>intermedius</u> 581	<u>melaninogenicus</u> 15930
Todd-Hewitt Broth (BBL)	21.4	6.2	0.15
Trypticase Soy Broth (BBL)	4.6	2.6	0.45
Brain Heart Infusion Broth (BBL)	2.3	1.85	0.01
Nutrient Broth (Difco)	N.G.[c]	N.G.	N.G.

a   Release of Azo dye from the collagenous proteolytic substrate
    Azocoll (Calbiochem) as compared to a Clostridiopeptidase A standard

b   Cultures were grown at 37°C under anaerobic conditions (modified GasPak
    system, BBL)

c   N.G. = no growth

shown). Because of the optimal collagenolytic activity in enrTHB, this medium was employed in all further experiments.

B. Growth characteristics

The characteristic growth patterns of B. asaccharo-lyticus and B. melaninogenicus subspecies were determined in order to identify the specific growth phases of each organism. These growth phases represent the immediate metabolic activity of an organism and will be used in later studies.

Typical growth curves of B. asaccharolyticus, B. melaninogenicus subsp. melaninogenicus and B. melaninogenicus subsp. intermedius are shown in Figure 1, 2 and 3 respectively.

C. Additives

In vivo, many anaerobic organisms, like the BPPOB, require metabolic endproducts which they receive from synergistic relationships with other microorganisms. In vitro, these substances must be supplied to the organisms to ensure optimal growth, although this addition does not truly recreate their normal environment. Traditionally, additives such as yeast extract, hemin and menadione have been used for the growth of BPPOB, although the effect of these additives on collagenolytic activity of oral Bacteroides has never been tested.

In this study, yeast extract, hemin, and menadione were tested seperately and in combination for their effect on

Figure 1.   Representative growth curve of <u>Bacteroides</u>

<u>asaccharolyticus</u> strain 381 in enrTHB at 37°C

under anaerobic conditions.   Each point

represents the mean viable count of duplicate

samples as described in Materials and Methods.

Figure 2.   Representative growth curve of <u>Bacteroides</u>

<u>melaninogenicus</u> subsp. <u>intermedius</u> strain

581 in enrTHB at 37°C under anaerobic

conditions. Each point represents the mean

viable count of duplicate samples as

described in Materials and Methods.

Figure 3. Representative growth curve of <u>Bacteroides</u>

<u>melaninogenicus</u> subsp. <u>melaninogenicus</u>

strain 15930 in enrTHB at 37°C under anaerobic

conditions. Each point represents the mean

viable count of duplicate samples as described
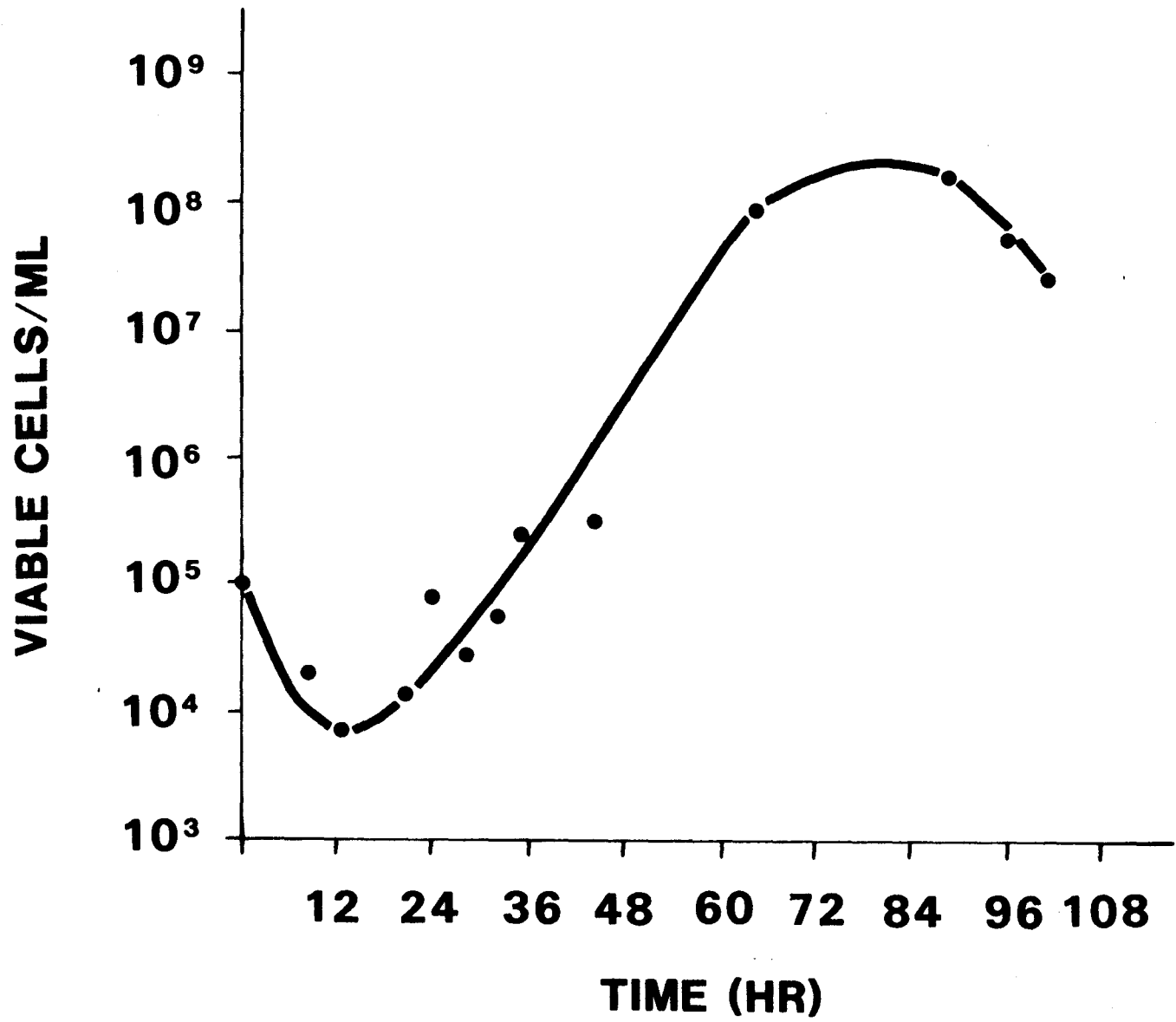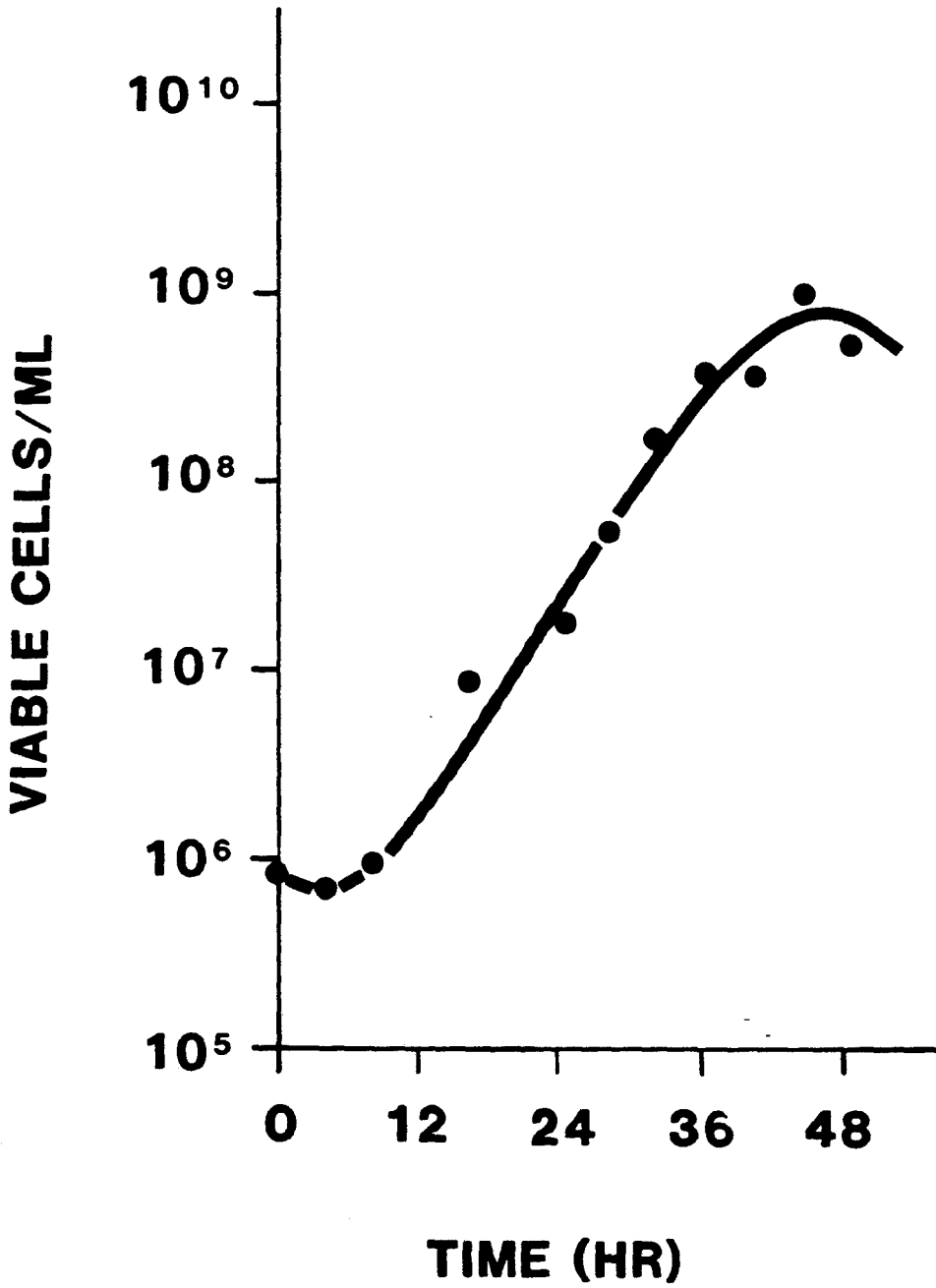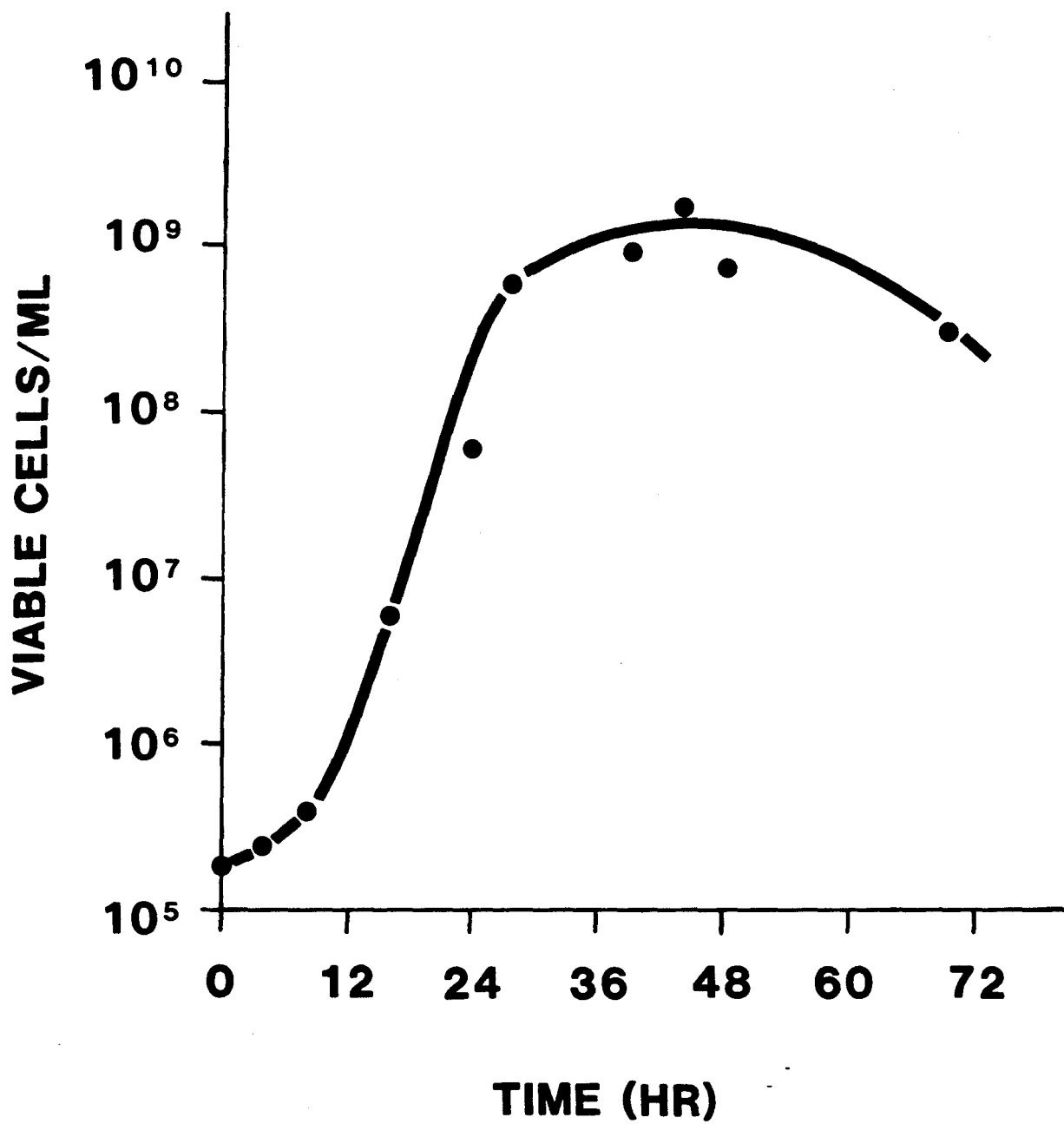
in Materials and Methods.

collagenolytic activity and described in the Materials and
Methods. As shown in Table II. B. asaccharolyticus and
B. melaninogenicus subsp. intermedius displayed
increased collagenolytic activity, as compared to the THB
alone, when grown in media containing hemin and menadione.
In contrast, the addition of yeast extract, alone, although
stimulating the growth of B. asaccharolyticus and B.
melaninogenicus subsp. intermedius depressed these
organisms' collagenolytic activity. This potentially
result would indicate that the use of yeast extract for in
vitro monitoring of collagenolytic activity by these BPPOB
could give misleading results. For this reason YE was not
used as an additive in any further studies unless otherwise
stated. B. melaninogenicus subsp. melaninogenicus,
not shown here, produced an extremely low level of activity
in all cases.

II. Collagenolytic Activity of BPPOB

Various strains of B. melaninogenicus subspecies
were screened for collagenolytic activity as described in
the Materials and Methods. As shown in Table III, B.
asaccharolyticus strain 381 and 382 displayed marked
collagenolytic activity as did B. melaninogenicus subsp.
intermedius strains 581 and 581B. B. melaninogenicus
subsp. intermedius strain 379 and 25261 and B. melanino-
genicus subsp. melaninogenicus strain 15930 displayed almost
no collagenolytic activity. As a result, it was determined
to use only BPPOB strains 381, 581, and 15930 for this study.

TABLE 2

Effect of Various Additives on the Collagenolytic Activity[a]
of <u>Bacteroides melaninogenicus</u>[b] Subspeices

Addition	Collagenolytic Activity (units/mg dry weight	
	Subspecies/Strain	
	<u>asaccharolyticus</u>/381	<u>intermedius</u>/581
None	1.8	0.9
Yeast Extract (0.5%)	0.8	0.4
Hemin (0.05%) and Menadione (0.0005%)	2.3	N.D.
Yeast Extract, Hemin and Menadione	2.25	1.35

a   Release of Azo dye from the collagenous proteolytic substrate, Azocoll (Calbiochem) as compared to a Clostridiopeptidase A standard.

b   All strains were grown in enriched Brain-Heart Infusion broth (BBL) for 9d at 37°C under anaerobic conditions, (modified GasPak system, BBL).

TABLE 3

Collagenolytic Activity[a] of <u>Bacteroides melaninogenicus</u>
Subspecies After 9d Growth[b] in <u>Enriched Todd-Hewitt Broth</u>

B. melaninogenicus Subspecies	Strain	Collagenolytic Activity (units/10.0 ml supernatant)
<u>asaccharolyticus</u>	381	20.5
<u>intermedius</u>	581	17.5
<u>melaninogenicus</u>	15930	<0.01

a   Release of Azo dye from the collagenous proteolytic substrate Azocoll as compared to a Clostridiopeptidase A standard.

b   Cultures were grown at 37°C under anaerobic conditions (modified GasPak system, BBL).

Gibbons and MacDonald (29) have compared the
collagenolytic activity of undertermined subspecies of B.
melaninogenicus during the characteristic growth phases.
However, these authors failed to standardize the colla-
genolytic activity of these organisms with their growth,
giving a false representation of these organisms activity
over time.

In this study the collagenolytic activity of B.
asaccharolyticus, B. melaninogenicus subsp. inter-
medius and B. melaninogenicus subsp. melaninogenicus,
expressed in units/mg dry weight, were compared to the
characteristic growth patterns of each organism as described
in Materials and Methods. As seen in Fig. 4 B.
asaccharolyticus and B. melaninogenicus subsp. inter-
medius behave similarly. B. melaninogenicus subsp.
melaninogenicus although displaying a normal growth
pattern, exhibit extremely low levels of collagenolytic
activity allowing no correlations to be made to its growth
pattern.

In both B. asaccharolyticus and B. melanino-
genicus subsp. intermedius activity remained extremely
low until bacterial dry weight began to increase sharply.
Collagenolytic activity peaked at 52 hours for subspecies
intermedius and at 56 hours for B. asaccharolyticus,
while bacterial growth reached its peak at 76 hours.
Collgenolytic activity could be detected only after
logarithmic phase had begun and these organisms had reached
a high level of metabolic activity. As the stationary

Figure 4. Collagenolytic activity of BPPOB as related to
representative growth curves. Predetermined
numbers of 18 h. old microorganisms were
inoculated to THB with 0.5% yeast extract and
incubated at 37°C under anaerobic conditions.
At each time period, a tube was removed and
culture supernatant tested for collagenolytic
activity as described in Materials and Methods.
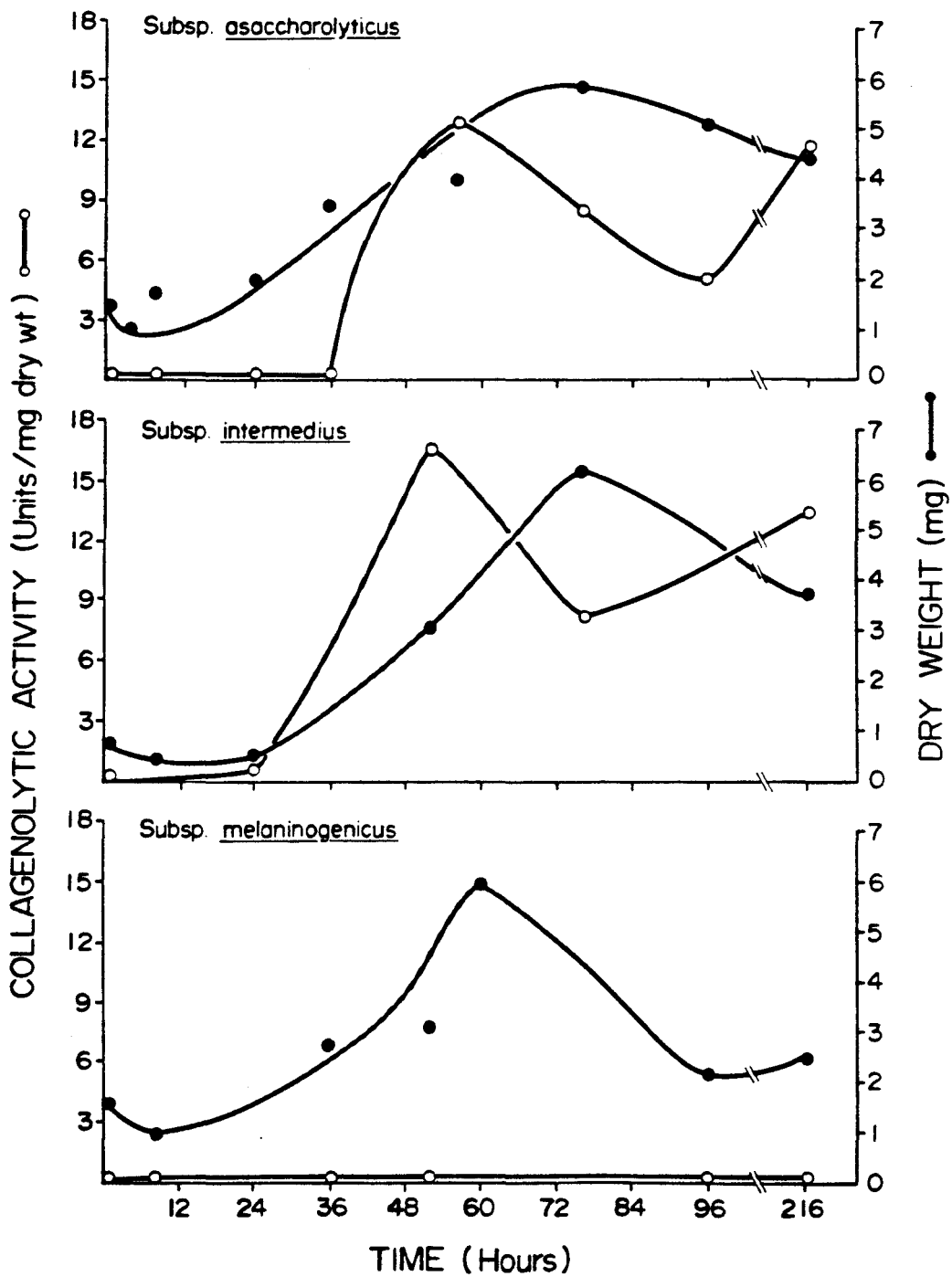
phase of growth was reached, collagenolytic activity began to drop, extracellular collagenolytic enzymes appear to be loosing activity under the growth conditions employed. Once the death phase occured, as indicated by a drop in dry weight, collagenolytic activity again rose, signifying that active enzymes were also released on autolysis of cells. It is quite clear that high levels of collagenolytic activity will be obtained on active growth of cells as well as when they lyse and dye during the continuous culture conditions of the oral cavity.

III. The Effect of Oral Conditions on the Collagenolytic Activity of BPPOB

The oral cavity is an extremely complex and variable environment. Many factors within the oral cavity may effect the collagenolytic activity of one of the BPPOB. In this study, the effect of pH and one of the major components of the saliva, Parotic Gland Secretion (PGS) on the collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subspecies was tested.

A. Determination of the optimal pH for the production of collagen degrading substances

The optimal pH for the collagenolytic activity was found to be over the range of from pH 7.0 - 7.4. More acid or alkaline conditions inhibited collagenolytic activity completely.

B. The effect of PGS on the collagenolytic activity of BPPOB subspecies

As seen in Fig. 5 the effect of PGS on the collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subspecies was varied. PGS had a stimulatory effect on the collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subsp. melaninogenicus while inhibiting this activity in B. melaninogenicus subspecies intermedius. Maximum stimulation of collagenolytic activity was observed for B. asaccharolyticus and B. melaninogenicus subsp. melaninogenicus when 0.1 ml or 1% PGS was added to the medium. When the PGS was increased to 1.0 ml or 10% of the medium, 95-99% of this stimulation was lost. In contrast, PGS had an inhibitory effect on the collagenolytic activity of B. melaninogenicus subps. intermedius. Maximum inhibition occured when 0.5 ml or 5% PGS was added to the medium. When the amount of PGS was decreased to 0.1 ml or 1% of the medium, 99% of this inhibition was lost. Similarly, when the amount of PGS was increased to 1.0 ml or 10% of the medium, 75% of this inhibition was lost.

The inhibition of collagenolytic activity of B. melaninogenicus subsp. intermedius is an interesting finding. Whether the same substances responsible for the stimulation of collagenolytic activity seen with B. asaccharolyticus and B. melaninogenicus subsp. melaninogenicus are responsible for the inhibition seen here is unknown.

Figure 5. Effect of parotid gland secretion (PGS) on
collagenolytic activity of black pigment-
producing oral <u>Bacteroides</u>.
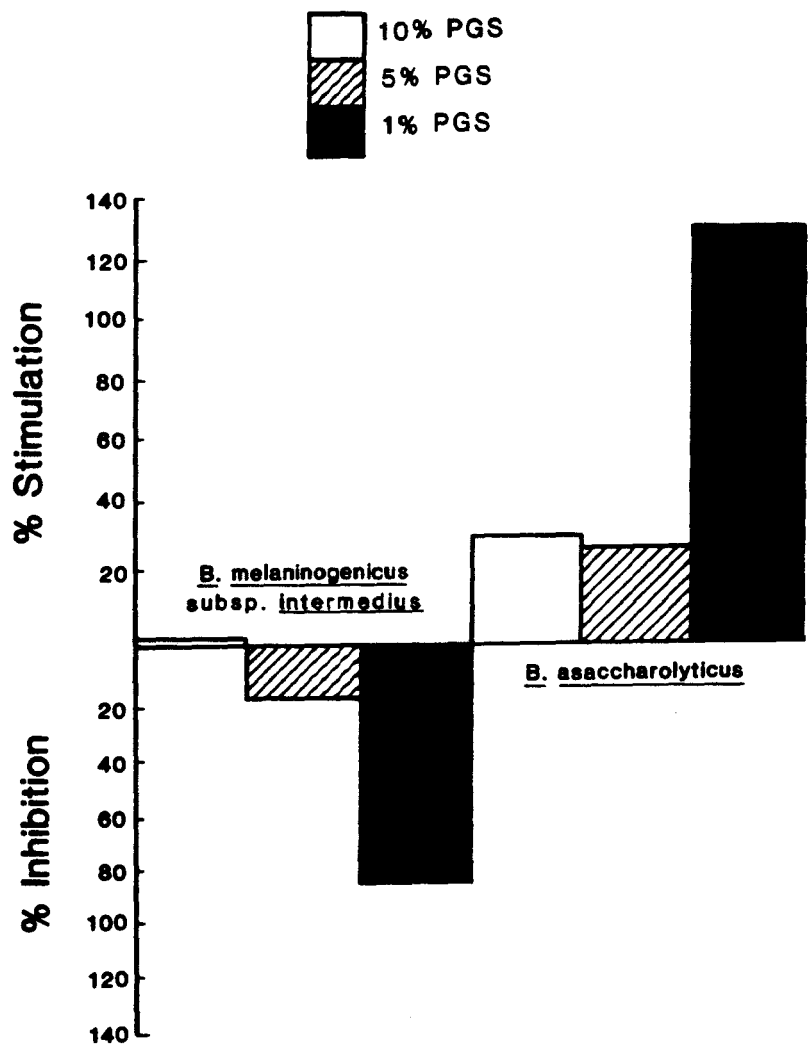
IV. Electron Microscopy

Although the collagenolytic activities of B. asaccharolyticus and B. melaninogenicus subspecies may play a major role in these organisms pathogenicity, it is also of great importance to know if these bacteria possess the ability to remain at the site of infection and prevent host defense mechanisms. One such way in which bacteria might accomplish this end is to produce some type of extracellular material, such as capsular material, matrix or fibrils. In order to gain some insight as to this ability, a preliminary electron microscopic study on the ultrastructure of B. asaccharolyticus and B. melaninogenicus subspecies was performed.

Seen in Fig. 6, 7, and 8 is a representative thin sections of (6) B. asaccharolyticus, (7) B. melaninogenicus subspecies melaninogenicus and (8) B. melaninogenicus subspecies intermedius. As shown, an electron dense material (EM) was consitently observed surrounding cells of B. asaccharolyticus.

In contract, B. melaninogenicus subsp. melaninogenicus possesses distinct fibrils. These type of fibrils classically play a role in an organisms ability to attach. The potential abilities of this organism to stay at the sight of infection, along with its stimulated increase in collagenolytic activity by PGS could greatly enhance its pathogenic potential. B. melaninogenicus subsp. intermedius does not possess any extracellular matter. Even

Figure 6. Electron microscopy of <u>B. asaccharolyticus</u>.

Bacteria were grown in enrTHB at 37°C under

anaerobic conditions for 9 days and then fixed

and embedded and sectioned as described in

Materials and Methods. The 1.5 cm bar represents

1μ.
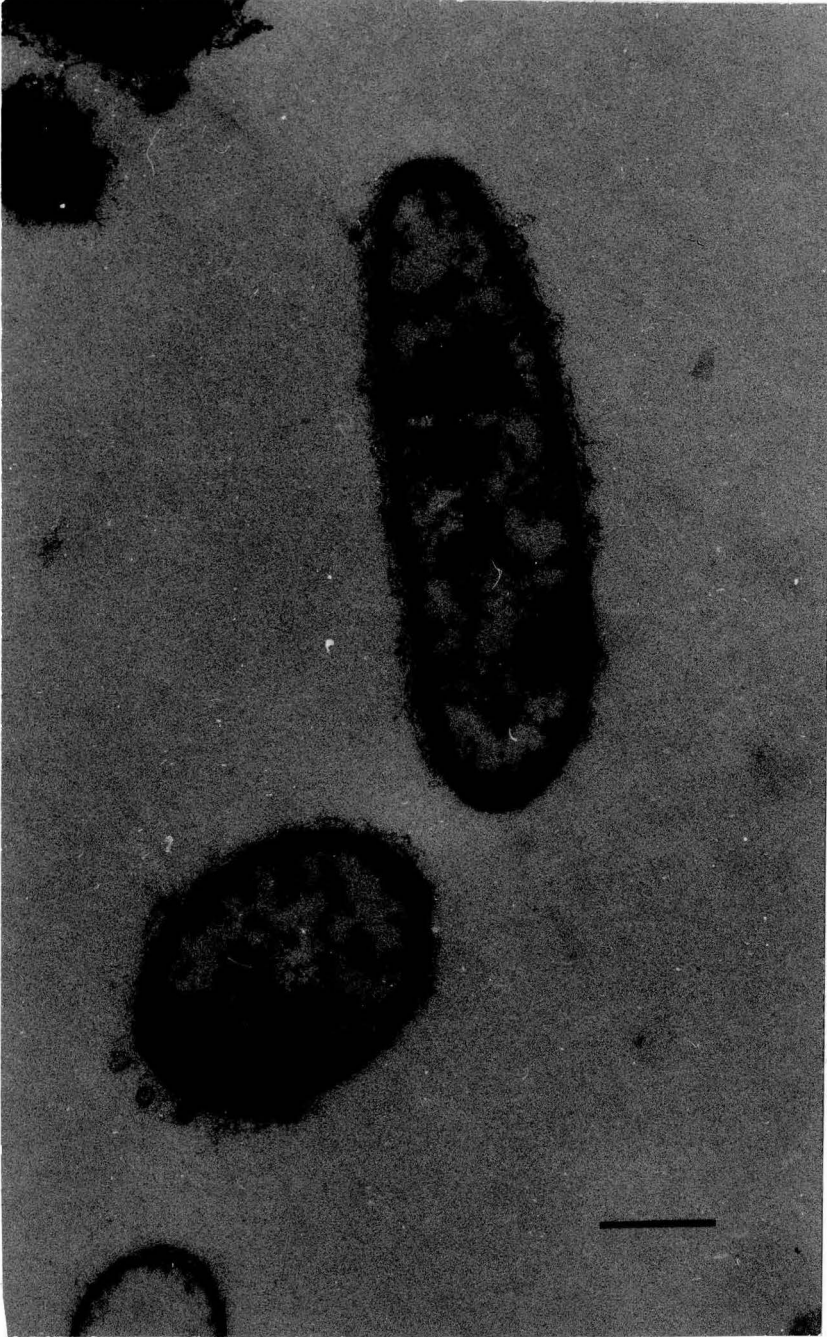
Figure 7. Electron microscopy of <u>B. melaninogenicus</u>
subsp. <u>melaninogenicus</u>. Bacteria were grown
in enrTHB at 37°C under anaerobic conditions
for 9 days and then fixed and embedded and
sectioned as described in Materials and Methods.
The 1.5 cm bar represents 1μ.
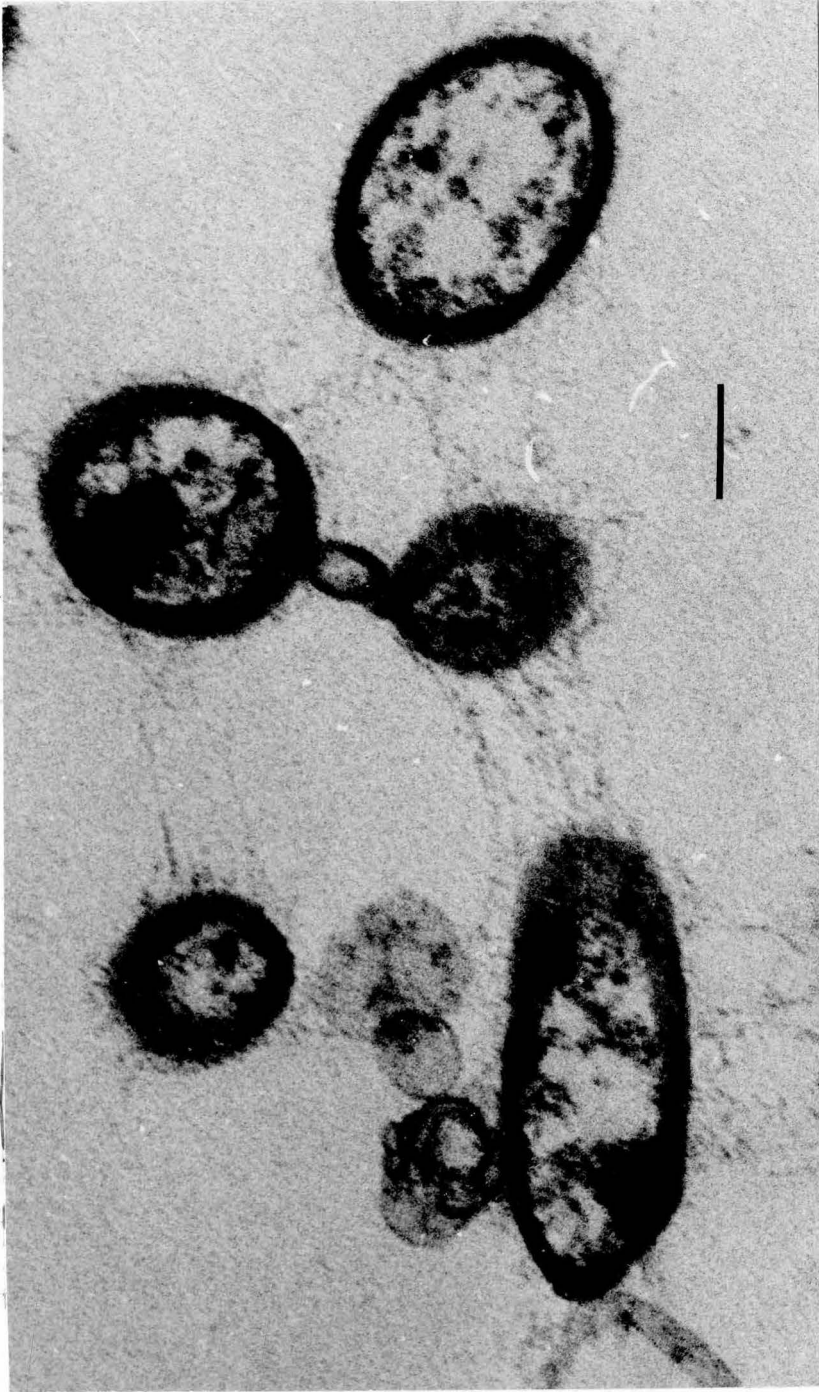
Figure 8. Electron microscopy of <u>B.</u> <u>melaninogenicus</u>

subsp. <u>intermedius</u>. Bacteria were grown in

enrTHB at 37°C under anaerobic conditions for

9 days and then fixed and embedded and sectioned

as described in Materials and Methods. The 1.5

cm bar represents 1μ .
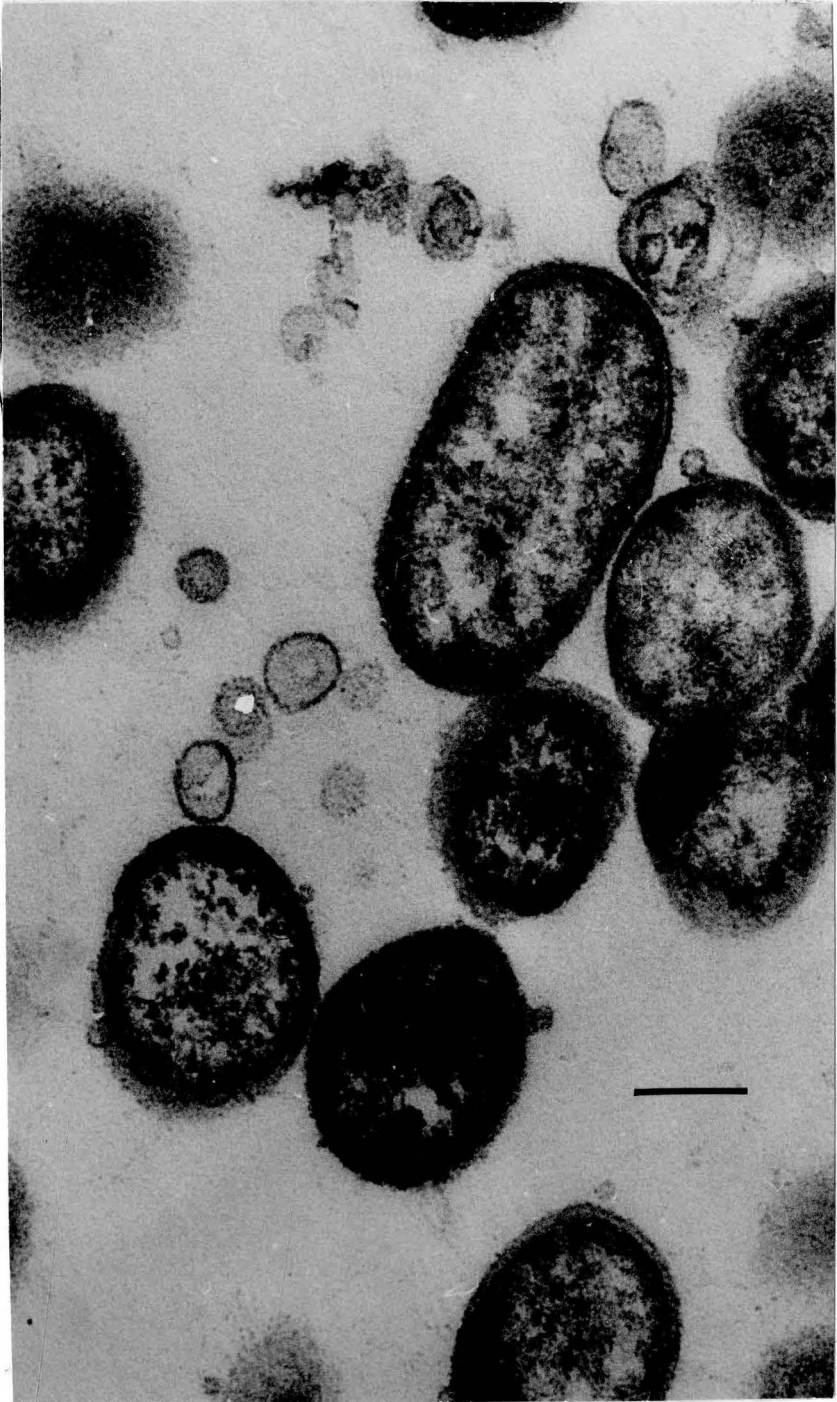
though this organism displayed a relatively high level of collagenolytic activity, its overall pathogenicity may be decreased due to the abscence of an attachment mechanism.

CHAPTER 4

DISCUSSION

The species <u>Bacteroides melaninogenicus</u> was first

described by Oliver and Wherry in 1921 (85). This species

contains strictly anaerobic non-motile, non-sporing,

gram-negative coccobacilli which produce dark brown

pigmented colonies on media containg blood. In 1973 Moore

and Holden (83) were able to divide the dark brown pigmented

<u>Bacteroides melaninogenicus</u> into three subspecies

<u>Bacteroides melaninogenicus</u>, subsp. <u>melaninogenicus</u>,

subps. <u>intermedius</u> and subsp. <u>asaccharolyticus</u>. Since

1973 much information has accumulated, both biochemical and

physiological, (42) amplifying the differences between each

subspecies. In addition, it has been observed that of the

BPPOB, <u>B. asaccharolyticus</u> is most frequently isolated

from clinical infections, including inflammatory periodontal.

The distinct biochemical, physiological and clinical

differences between the three subspecies provoked the ICSB

taxonomic subcommittee on gram-negative anaerobic rods to

suggest that <u>B. melaninogenicus</u> subsp. <u>asaccharolyticus</u>

was sufficiently different from the other subspecies to be

regarded as a separate species designated <u>Bacteroides</u>

<u>asaccharolyticus</u> (3).

Prior to 1973, much research was performed implicating

<u>B. melaninogenicus</u> as an essential pathologic component

of inflammatory periodontal disease (29,38,59,60,110) Un-
fortunately, this research was performed without knowing
which of the species or subspecies of <u>Bacteroides</u> was
involved.

There is currently much controversy as to which
microorganisms are isolated from different stages and types
of periodontal disease. However, it is clear that <u>B.</u>
<u>asaccharolyticus</u> is the most frequently isolated micro-
organism from advanced periodontitis. In a recent study,
White and Mayrand (124) found that, although <u>B.</u>
<u>melaninogenicus</u> subps. <u>intermedius</u> was frequently
isolated from pockets of depth 4 to 6 mm, <u>B.</u>
<u>asaccharolyticus</u> was still the most frequently isolated
BPPOB from pockets of 6 mm or greater. In addition, they
stressed the need to characterize the pathogenic properties
of <u>B. asaccharolyticus</u>. The recent accumulation of
information illuminating the biochemical, physiological and
clinic differences between each organism has made it
essential to reevaluate previous information presented on
<u>B. melaninogenicus</u>.

In our laboratory it was shown that oral strains of
<u>B. asaccharolyticus</u> displayed consistently high
collagenolytic activity. <u>B. melaninogenicus</u> subsp.
<u>intermedius</u> revealed marked collagenolytic activity in
comparision to <u>B. melaninogenicus</u> subsp. <u>melanino-
genicus</u> which exhibited extremely low collagenolytic
activity. This data coincides with a study (68) which
surveyed the collagenolytic activity <u>B. asaccharolyticus</u>

and B. melaninogenicus subspecies using radioactively
labeled bovine achilles tendon in an assay described by
Gisslow and McBride (1975).

The potential importance of an enzyme such as this
has been described above and elsewhere (91,69,115,121,53).

Once the collagenolytic activity of B.
asaccharolyticus and B. melaninogenicus subspecies was
established it was of considerable interest to determine
when this activity was expressed during the characteristic
growth patterns of these organisms. An obvious difference
in the collagenolytic activities of these organisms might
partially explain why B. asaccharolyticus is most
frequently isolated from clinical infections. When
collagenolytic activity was compared to bacterial growth
patterns, B. asaccharolyticus and B. melaninogenicus
subsp. intermedius behave similarly. B.
melaninogenicus subsp. melaninogenicus although dis-
playing a typical growth pattern, exhibited extremely low
amounts of collagenollytic activity.

It was interesting to note that in both B.
asaccharolyticus and B. melaninogenicus subsp.
intemedius collagenoltyic activity remained extremely low
until bacterial dry weight began to increase sharply. Only
after cells reached a high metabolic activity, during
logarithmic phase, could collagenolytic activity be detected.
This would indicate that collagenolytic activity is present
in actively growing cells.

It is clear that high levels of collagenolytic activity are present while cells are actively growing as well as when they die and lyse in the continuous culture conditions of the oral cavity. This data would indicate that the enzyme produced by B. asaccharolyticus and B. melaninogenicus subsp. intermedius is being released from the cell and is not necessarily dependent upon cell lysis for its entry into the environment.

In contrast, MacDonald and Gibbons, (29) found that the collagenolytic activity of B. melaninogenicus could not be seen until stationary phase was reached and culture autolysis began. Similarly, these authors observed that collagenolytic activity increased as autolysis continued. Gibbons and MacDonald (29) suggested that the enzymes responsible remained intracellular until released by autolysis. Unfortunately, these authors failed to standardize collagenolytic activity of B. melaninogenicus by equilibrating their values with the quantity of bacteria present. Instead they expressed their collagenolytic determinations in ranges, making definitive interpretation of this data impossible.

In a recent study, Robertson et al. (90) demonstrated that as Bacteroides reaches stationary phase, collagenolytic activity is seen in the media, presumably reflecting auto-lysis. Collagenase has been detected in both culture media and cell sonicates (90,128). It is now generally accepted that although collagenase may be detected in culture supernates, the enzymes is actually cell associated (90,69,

68,94). Regardless of the specific time course of
production, there is no doubt a large bulk of lysing and dead
cells in the subgingival plaque. This material could provide
large stores of the collagenolytic enzymes for tissue
destruction. The data described above (Fig. 4) clearly
indicates that B. melaninogenicus subsp. melanino-
genicus does not produce appreciable levels of collagenase
under our experimental conditions. In contrast to this,
Robertson et al. found that all species of Bacteroides
tested, including all B. melaninogenicus and B.
fragilis, did exhibit collagenolytic activity. Mayrand
(69) demonstrated that formerly B. asaccharolyticus
but not B. melaninogenicus produced collagenase.
Steffen and Hentges (115) showed that all oral Bacteroides
but not B. fragilis produce the enzyme. Most recently,
using a slightly different set of assays and procedures,
Mayrand et al. (70) have indicated that only B.
gingivalis possess collagenolytic activity. The dis-
crepancies seen between these studies most likely is due to
the variety of strains tested, environmental conditions used,
and the sensitivity of the assays. It is important to note
that in our study and those of others, the end result of
collagen breakdown is due to the combined action of bacterial
collagenase and other proteolytic enzymes produced by these
microorganisms. In fact, Yamamota (128) demonstrated that
B. gingivalis produces a somewhat unique proteolytic
enzymes not seen in other Bacteroides species. Sobhy
et al. (107) concluded due to findings from clinically

diseased tissue that both collagenase and proteases were important in the gingival matrix destruction seen in periodontal disease.

The human periodontal pocket, initial site of inflammatory periodontal disease contains fifty or more species of microorganisms (45). The bacteria which inhabit the mouth constitute a ecosystem which has physical, chemical and biological properties that not only dictate the composition of the community, but determines which organisms will dominate the system and which will fail to survive. The biological relations of microorganisms are of the greatest significance in the economy of nature and in the production of disease.

Many factors play a role in the development of clinical infection, yet investigators have concluded that infections caused by several organisms are not microbially specific, but are specific in the biochemical sense (1). The initiation of disease depends not only a specific combination of microbes, but also on the elaboration of a specific combination of require metabolites (69). Research done by MacDonald et al. (59) found that the occurence of <u>Bacteroides</u> <u>melaninogenicus</u> in guinea pig infections was dependent on the elaboration of Vitamin K like compounds elaborated by other organisms. In this respect, it seemed evident that the elaboration of metabolites by other organisms (along with the natural environment produced by the host in the oral cavity) do effect the pathogenic potenitalities of <u>Bacteroides</u> <u>asacharolyticus</u> and other BPPOB. However, the intricacy

and complexity of the nutritional and physiological environment of the periodontal pocket, the site of IPD, can not be reproduced in vitro. The synthetic environment employed in the growth and maintenance of the BPPOB in vitro could alter the metabolic and physiologic patterns of these organisms, ultimately altering their pathogenic qualities.

In this study it was found that the addition of yeast extract, a normal constituent of the "complex media" used to maintain fastidious microorganisms like the BPPOB, although stimulating the growth of B. asaccharolyticus and B. melaninogenicus subspecies, depressed these organisms collagenolytic activity. The specific substance or substances capable of effecting these BPPOB was not established. It is possible that the overall increase in the numbers of peptides or amino acids added to the media through the yeast extract may act as feedback repressors of collagenase synthesis. A similar explanation was given by MacDonald et al. (61) when they discovered that diluting media employed to grow B. melaninogenicus (thus decreasing the overall peptide and amino acid content) decreased growth of organisms while strikingly increasing their collagenolytic activity. More recently, Robertson et al. found that when strains of Bacteroides and Actinobacillus were grown in a peptide depleted medium their enzyme activities were enhanced. No explaination was given (91). This is significant in that a similar explanation could account for the enhancement of the pathogenicity of B. asaccharolytics and B. melaninogenicus subspecies in the presence of other

suitable organisms. If these organisms were found to utilize the endproducts of collagenolytic activity, thus decreasing the peptide and amino acid content of the environment, they could mediate the collagenolytic activity of BPPOB by removing these feedback repressors from the environment.

It is of considerable importance to understand the effect of in vitro conditions which may alter the pathogenic qualities of B. asaccharolyticus and B. melanino-genicus subspecies. Any in vitro conditions which can alter the collagenolytic activity of these organisms will also alter our understanding of how these BPPOB behave in their natural environment. In order to gain some knowlege of how the collagenolytic activity of these organisms is effected by the intricate and complex nutritional and physiological environment of the periodontal pocket, pH and PGS were tested. By definition, collagenase are proteolytic enzymes capable of degrading native collagen fibrils under physiologic conditions of pH and temperature. Since the physiologic temperature of the oral cavity is normally constant at 37°C, it was necessary to keep this temperature during all experimental studies on the collagenolytic activity of these BPPOB. In contrast, the physiologic pH of the oral cavity is extremely variable. In order to determine if B. asaccharolyticus and B. melaninogenicus sub-species are active at the pH of the periodontal pocket, pH 7.4, the effect of various pH on the collagenolytic activity of these organisms was tested. In agreement with other authors (29,35) collagen breakdown was greater over a pH of

7.0 to 7.4. More acidic conditions inhibited collagenolytic
activity completely. The fact that collagenolytic activity
is seen at pH 7.4 in B. asaccharolyticus and B.
melaninogenicus subspecies suggest that it is related to
the collagen destruction seen in periodontal disease.

The most obvious environmental factor present in the
oral cavity which may have an effect on the collagenolytic
activity of B. asaccharolyticus and B. melanino-
genicus is the saliva. Saliva is a mixed secreation more
than 90% of which is produced by the parotid, submandibular
and sublingual glands. The remainder is contributed by
accesory glands present on the soft palate and on the
internal surface of the lips and cheeks. In addition, it
has been estimated that between 700 and 800 ml of saliva
are produced daily. Saliva has many functions of which the
most important are protection, digestion, control of water
balance and lubrication. The pH of the saliva varies from
5.6 to 8.0 and contains considerable amounts of Ca^{++}.

One primary means by which such collagenolytic activity
might be controlled or altered is by salivary components.
Protein (1.8 - 4.2 g/l) and amino acids (40 mg/l) are major
components of saliva (45) and may have an effect on colla-
genolytic activity. Some amino acids have been found to
inhibit elaboration of collagenase. Monboisse (80) noted in
his study of Acinetobacter that these materials did, in fact,
repress synthesis of collagenase. MacDonald et al. (60)
reported that when their growth media for Bacteroides was
diluted, cultures grew slower, but collagenase activity was

greatly increased. P.B. Robertson et al. (91) found that the collagenolytic activity of stains of Bacteroides and Actinobacillus were enhanced when grown in a peptide depleated medium. Yeast extract in our study was demonstrated to have essentially the same effect. Proteinaceous components might well be responsible.

Although saliva plays an obvious role in both the environmental and physiological conditions of the oral cavity their has been no information accumulated as to the effect of saliva on the collagenolytic activity of B. asaccharo-lyticus and B. melaninogenicus subspecies. One reason which might explain the lack of information on the effect of saliva on the collagenolytic activity of these organisms is the variability of the composition of saliva. Whole (or mixed) saliva, which is usually collected from the mouth during paraffin wax stimulation, not only contains fractions from each gland, but in addition contain food debris, plaque, bacteria, degraded mammalian cells and possibly some gingival sulcular fluid. The extremely variable composition of this fluid would make any explanation of its effect on the BPPOB impossible. For this reason, and because the parotid gland becomes the major contributor to mixed saliva under stimulated conditions (48) it was decided to test the effect of Parotid Gland Secretion, collected with special canniculi direclty from the Stenson's Duct. This material is pure and sterile and is truely representitive of the secretion of the gland in question. In addition, it has been shown that whole saliva contains vitamin K, which would have a direct effect

on the BPPOB (58). In contrast, the same author discovered that this growth factor was not present in PGS.

The effect of PGS on the collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subspecies was compared to identical cultures of each species containing equal amounts of H_2O. A potentially important observation was that PGS was able to stimulate collagenolytic activity in B. asaccharolyticus and B. melaninogenicus subsp. melaninogenicus. The constituent of the PGS which was able to impose such effects on the collagenolytic activities of these organisms was not determined. However, it is apparent that this factor is in no way related to the amino acid or peptide concentration of PGS, since the effect of PGS was compared to an identically diluted culture with distilled H_2O in place of the PGS. Futhermore, the high amino acid and peptide concentration of PGS (58) would be expected to decrease the collagenolytic activity of these organisms as was previously shown by Robertson et al. (91) and would be expected to have the same effect on all three organisms. In this light, it is possible that the PGS contains a substance which acts as an inducer, inactivating the feedback repression of the amino acids and peptides produced through the destruction of collagen. The most important observation which may be drawn from the data is that PGS has a variable effect on the three organisms. The normal study of the collagenolytic activity of these organisms is done in a complex media. Once a singular oral environmental factor is added to this media the organisms behave differently. This

is underlined by the fact that although B. melanino-
genicus subsp. melaninogenicus did not exhibit marked
collagenolytic activity in culture, once PGS was added its
activity increased substantially. In contrast B.
melaninogenicus subsp. intermedius which revealed marked
collagenolytic in culture, was substantially inhibited by the
addition of singular oral environmental factor. It is of
great importance in this study of the collagenolytic activity
of B. asaccharolyticus and B. melaninogenicus sub-
species to realize that oral environmental factors can
drastically alter the potential of these organisms to degrade
collagen.

Another factor which might play a role in the ability
of Bacteroides to initiate and/or participate in periodontal
disease would be surface structures such as capsules of
fibrils. Dahlen and Nygram (14) demonstrated a uniform
polysaccharide lining in all Bacteroides strains yet the
surface layer was least expressed in B. melaninogenicus
(in about 10% of the cultures examined). Other investigators
have also demonstrated a capsule by ruthenium red staining
(46,64,54,65,76,126). As seen in Fig. (6, 7, and 8) our
studies have shown that an electron dense material (EM) was
consistenly observed surrounding cells of B. asacchar-
olyticus. In a contrast, B. melaninogenicus subsp.
melaninogenicus was shown to possess distinct fibril
B. melaninogenicus subsp. intermedius did not possess
any extracellular material. Capsuler material or surface
structures may play a role in pathogenesis of mixed

infections. Coaggregation between <u>Actinomyces</u> <u>viscosus</u> and <u>Bacteroides</u> <u>gingivlis</u> could not be accounted for only by the fimbriae of <u>Actinomyces</u>. Specific surface structures of <u>Bacteroides</u> <u>gingivalis</u> were proposed as playing a major role in the process. The types of fibrils observed with <u>Bacteroides</u> <u>melaninogenicus</u> subsp. <u>melaninogenicus</u> classically play a role in an organisms ability to attach. The potential abilities to stay at the sight of infection, along with this organisms increased collagenolytic activity in the presence of PGS could greatly enhance its pathogenicity. In contrast, even though the <u>Bacteroides</u> <u>melaninogenicus</u> subsp. <u>intermedius</u> displayed a relatively high level of collagenolytic activity, the absence of any extracellulr material may decrease its overall pathogenicity.

CHAPTER V

SUMMARY

Characterization of collagenase production by black-pigment producing oral bacteroides (BPPOB) was investigated. Bacterial cells were grown at 37°C under anaerobic conditions in enriched Todd-Hewitt Broth. In this study, when yeast extract, hemin and menadione were tested seperately and in combination for their effect on collagenolytic activity B. asaccharolyticus and B. melaninogenicus subspecies intermedius displayed increased collagenolytic activity, as compared to Todd-Hewitt Broth alone, when grown in media containing hemin and menadione. In contrast, the addition of yeast extract, alone, though stimulating the growth of B. asaccharolyticus and B. melaninogenicus subsp. intermedius depressed these organisms collagenolytic activity. Collagenase level was determined using the collagenous substrate Azocoll in a colorimetric assay and employing a standard curve of Clostridiopeptidase A.

B. asaccharolyticus and B. melaninogenicus subspecies intermedius displayed marked collagenolytic activity, while activity was not observed in subspecies melaninogenicus. When collagenolytic activity was compared to bacterial growth patterns it was found that the highest

level of activity occured on active growth of cells as well as during cell death and lysis.

When certain oral environmental conditions were tested for their effect on the collagenolytic activity of BPPOB it was found that the optimal pH for production of collagenolytic substances was 7.0 - 7.4. When Parotid Gland Secretion was added to the growth media of the BPPOB both inhibition and stimulation of collagenolytic activity occured. Parotid Gland Secretion greatly stimulated collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subsp. melaninogenicus (activity not detectable under other conditions) and inhibited collagenolytic activity B. melaninogenicus subsp. intermedius.

Electron microscopy revealed that B. asaccharolyticus and B. melaninogenicus subspecies melaninogenicus possed extracellular substances.

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APPROVAL SHEET

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The final copes have been examined by the director of the thesis
and the signature which appears below verifies the fact that any
necessary changes have been incorporated and that the thesis is
now given final approval by the Committee with references to
content and form.

The thesis is therefore accepted in partial fulfillment of the
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