DEGRADATION OF COLLAGENOUS SUBSTRATES BY *BACTEROIDES MELANINOGENICUS*[1]

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It is well known that certain *Clostridium* species produce proteolytic enzymes capable of hydrolyzing native collagen, but apparently other bacteria possessing such enzymes have not been reported. Organisms with this capacity have been sought unsuccessfully in traumatic wounds (MacLennen, Mandl, and Howes, 1953), among salt tolerant bacteria (Everett and Cordon 1955), and in the oral cavity of man (Roth and Meyers, 1956; Schultz-Haudt and Scherp, 1955; Mergenhagen and Scherp, 1960). It has seemed likely that collagenolytic organisms are among the members of the oral microbiota since collagen degradation is a feature of both dentinal caries (Engel, 1950) and periodontal disease (Lucas and Thonard, 1955). *Clostridium* species are absent from the oral cavity or present only as transients. In addition, it has been reported that mixed cultures from the oral cavity can degrade native collagen (Mergenhagen and Scherp, 1960).

In the course of investigating the basis for pathogenicity of certain oral bacteria capable of producing a mixed anaerobic infection in guinea pigs, one bacterium, *Bacteroides melaninogenicus*, was found to hydrolyze native collagen. This paper presents the results of studies of this collagenolytic property.

MATERIALS AND METHODS

Cultures and cultural methods. Thirteen oral strains of *B. melaninogenicus* were isolated from gingival scrapings as previously described (Gibbons and Macdonald, 1960); 14 intestinal strains were isolated from anal swabs. Dr. M. Lev, University of Reading, England, kindly provided three rumen strains. All strains were found to require either hemin, menadione, or both for growth. They were maintained by weekly transfer in a medium of the following composition: trypticase, 17.0 g; yeast extract, 3.0 g; NaCl, 5.0 g; K_2HPO_4, 2.5 g; sodium thioglycolate, 0.5 g; hemin, 5 mg; $NaHCO_3$, 1.0 g; menadione, 0.5 mg; distilled water, 1,000 ml. The $NaHCO_3$ and menadione were sterilized by filtration, the remaining ingredients were autoclaved at 121 C for 15 min. This medium was employed for all studies reported.

Measurement of collagenolysis. Cultures were screened for collagenolytic activity using neutral salt extractable collagen as substrate. This was prepared from young guinea pig skins by the method of Gross (1958). Typical extracts contained approximately 0.2% collagen as judged by hydroxyproline content. The extracts were sterilized by millipore filtration in the cold, and 2.0-ml samples were dispensed in sterile culture tubes. These were immediately incubated at 37 C in a water bath until a gel formed. Five milliliters of the trypticase yeast extract medium were then added aseptically to each tube. The tubes were inoculated with strains of *B. melaninogenicus* and incubated in Brewer jars under 95% hydrogen and 5% carbon dioxide for 14 days at 37 C. The cultures were then centrifuged at 8,000 $\times$ *g* for 10 min to sediment bacteria and residual collagen. The residues were hydrolyzed in 6 N HCl for 4 hr at 121 C, and analyzed for hydroxyproline by the method of Neuman and Logan (1950). The 40 C oxidation temperature recommended by Leach (1960) improved reproducibility and was employed routinely. Collagenolysis was determined by comparing the amount of hydroxyproline present in culture residues with that of uninoculated controls.

Hydrolysis of native collagen was demonstrated using either fresh or lyophilized cells obtained from a 48- to 72-hr broth culture. A suspension containing approximately 30 mg dry weight of cells per ml was prepared in 0.067 M phosphate buffer (pH 7.2), and 4.0-ml samples were added to segments of fresh untreated rat

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tail. The mixture was overlaid with 0.5 ml toluene to prevent microbial growth, and incubated in stoppered tubes at 37 C for 3 days. After incubation, the mixture was centrifuged at 20,000 × *g* for 10 min, and samples of the supernatant fluid were removed for hydroxyproline analysis. These were hydrolyzed with an equal volume of 12 N HCl in sealed tubes for 4 hr at 121 C. Because of the large amount of extraneous material in the hydrolyzates resulting from cell autolysis, it was necessary to employ the ether extraction procedure of Mitoma et al. (1959) for analysis of hydroxyproline. The amount of hydroxyproline released from the rat tail was compared with that of heat inactivated controls to determine collagen hydrolysis. Rat tail segments exposed to trypsin (Nutritional Biochemicals Corporation), 1 mg per ml, served as additional controls.

To quantitatively determine collagenolytic activity, a relatively pure homogeneous collagen substrate was required. Collagen prepared by citrate extraction of guinea pig skins according to the procedure of Gallop (1955) was found suitable. This material assayed over 90% collagen as judged by its hydroxyproline content, and could be conveniently stored in lyophilized form. For use, sufficient collagen was suspended in cold 0.5 M acetic acid to make a 0.25% suspension. Continuous stirring at 4 C for 4 to 6 hr resulted in a colorless, viscous, opalescent solution. This was dialyzed against several changes of 0.2 M phosphate buffer (pH 7.2) to remove the acetic acid, and stored at 4 C prior to use.

Assays were performed by adding 2.0 ml of substrate solution to a 1.0-ml aliquot of the enzyme (either a cell-free enzyme preparation, or a suspension containing 30 mg dry weight of cells), plus any desired additives. Incubation was at 25 C to prevent gelling of the collagen. The reaction was stopped by the addition of sufficient trichloroacetic acid to make the final concentration 20%. The mixtures were centrifuged at 20,000 × *g* for 10 min and the supernatant liquors decanted. Samples of these were hydrolyzed in sealed tubes with 12 N HCl and analyzed for hydroxyproline. The production of hydroxyproline peptides was found to be proportional to enzyme activity over an incubation period of at least 90 min, or until more than 50% of the collagen was hydrolyzed. Collagenolytic activity was expressed as micrograms of collagen hydrolyzed (assuming hydroxyproline content of collagen to be 13%) per hr per 30 mg dry weight cells, or per ml cell-free enzyme preparation.

To assess the effect of pH on enzyme activity, acetic acid solutions of collagen were dialyzed against buffers covering a pH range of 3.5 to 8.5. Acetate buffers were employed in the range pH 3.5 to 5.5; phosphate buffers in the range pH 6.0 to 7.5; and borate buffers from pH 7.5 to 8.5. The ionic strength, which influences solubility of the collagen, was adjusted to 0.4 in all buffers by the addition of sodium chloride. Lyophilized cells at a concentration of 30 mg per ml in the appropriate buffer served as a source of enzyme. The pH of all reaction mixtures was measured.

Miscellaneous proteolysis tests. Gelatin hydrolysis was measured viscosimetrically according to the procedure described by Pelczar, Hansen, and Konetzka (1955). All samples were centrifuged at 20,000 × *g* for 10 min after being heat inactivated, and the supernatant liquor used for measurements. Azocoll (kindly supplied by J. J. Gilroy, Colorado State University) degradation was determined visually by observing dye release. Digestion of casein, egg albumin, and human plasma were demonstrated by the increase in nonprotein nitrogen resulting when these substrates were incubated with a suspension of the organisms.

RESULTS

Digestion of reconstituted collagen gels. All 30 strains of *B. melaninogenicus* grown in trypticase yeast medium for 14 days hydrolyzed reconstituted collagen gels. Strains varied in their activity, some degrading as little as 20% and others as much as 95%. In general, the oral strains were the most active, digesting an average of 55% of the collagen. Intestinal and rumen strains averaged 35 and 32%, respectively. A single strain (K 110) derived from the oral cavity and used in previously reported studies of mixed anaerobic infections in guinea pigs (Macdonald et al., 1956; Macdonald, Gibbons, and Socransky, 1960) was used for all other studies. This strain hydrolyzed from 40 to 90% of reconstituted collagen gels in 14 days in repeated experiments. Control cultures of *Clostridium histolyticum* were

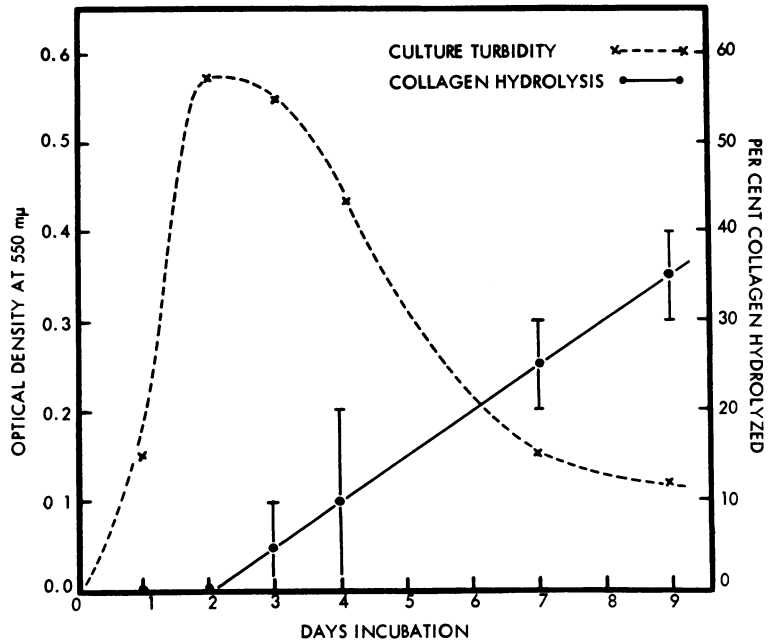

Fig. 1. Culture optical density and hydrolysis of reconstituted collagen gels by *Bacteroides melaninogenicus* measured over a 9-day period. The range of collagenolytic determinations is indicated by brackets.

significantly more active, completely hydrolyzing the collagen preparations in 48 hr.

It was observed, however, that cultures of *B. melaninogenicus* attained maximal growth within 48 to 72 hr, but that degradation of the collagen gel did not occur until culture autolysis began. This was verified by measuring culture turbidity and digestion of collagen gels over a 9-day period. Collagenolysis (Fig. 1) was not evident until the third day when culture autolysis began, and it increased as autolysis continued. This suggested that the enzyme responsible remained intracellular until released by autolysis.

Additional evidence of the intracellular nature of the enzyme was obtained by measuring the gelatinase activity of washed cells, and culture supernatant liquor derived from a 48-hr broth culture. Gelatin was employed as substrate on the assumption that the collagenolytic enzyme would hydrolyze it, and that the absence of gelatinase activity would indicate the absence of the collagenolytic enzyme. Similarly, Oakley, Warrack, and Warren (1948), working with *Clostridium welchii*, believed that a negative azocoll test meant the absence of collagenolytic activity, whereas positive tests were less meaningful. The results (Fig. 2) indicate that washed

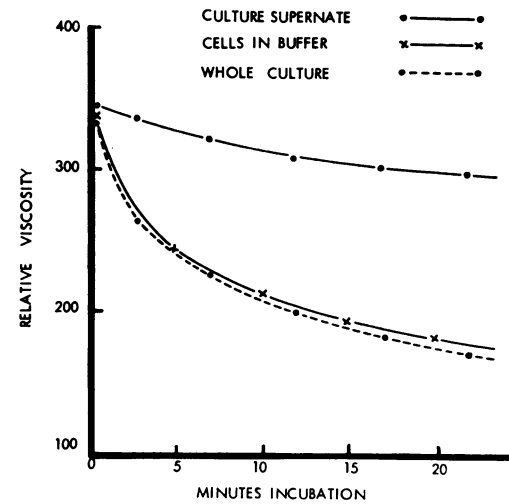

Fig. 2. Gelatin hydrolysis by cells, culture supernatant liquor, and whole cultures of *Bacteroides melaninogenicus.*

cells contain the bulk of gelatinase activity present in the whole culture, and that the culture supernatant liquor possesses little activity. Thus, little, if any, collagenolytic enzyme was present in the extracellular fluid.

Digestion of native collagen. Attempts to

TABLE 1

Hydrolysis of native collagen by Bacteroides melaninogenicus

Cell Preparation (30 mg Dry Wt per ml)	Total Hydroxyproline Released*
	μg
Fresh cells plus rat tail†	1,080
Heat inactivated fresh cells plus rat tail	80
Heat inactivated fresh cells alone	0
Lyophilized cells plus rat tail	880
Heat inactivated lyophilized cells plus rat tail	45
Heat inactivated lyophilized cells alone	0
Fresh cells plus gingival tissue‡	96
Heat inactivated fresh cells plus gingival tissue	<15

* Rat tail exposed to 1 mg per ml of trypsin released 110 μg of hydroxyproline.

† Segments of untreated rat tail weighing approximately 100 mg were employed.

‡ Normal human gingival tissue segments weighing approximately 20 mg.

demonstrate hydrolysis of untreated rat tail collagen by growing cultures of *B. melaninogenicus* were inconclusive. The amount of hydroxyproline released from the tail segments by cultures was only slightly higher than from uninoculated controls. The best results were obtained when cultures were incubated for 14 days with 100-mg segments of rat tail. Such culture supernatants usually contained 15 to 30 μg of hydroxyproline per ml. The possibility that the organisms were metabolizing released hydroxyproline was ruled out by growing them in broth containing 30 μg hydroxyproline per ml and analyzing the medium after incubation.

It therefore appeared that to demonstrate active hydrolysis of native collagen, it would be necessary to concentrate the enzyme. This was accomplished by preparing cell suspensions containing 30 mg dry weight per ml of fresh or lyophilized cells. When 4.0-ml samples of these were incubated with untreated rat tail, considerable collagenolysis occurred (Table 1). Segments (20 mg) of normal human gingival tissue, removed during periodontal treatment, were also used as a source of native collagen. These also were hydrolyzed by the organism. In addition to native collagen, *B. melaninogenicus* actively hydrolyzed azocoll, casein, egg albumin, and plasma protein.

Characteristics of the enzyme. Collagenolytic activity of whole cells against soluble collagen ranged from 870 to 1,250 μg collagen hydrolyzed per hr per 30 mg dry weight of cells. Lyophilization did not affect activity. When compared with *C. histolyticum*, *B. melaninogenicus* proved much less active. Culture filtrates of *C. histolyticum* were found to hydrolyze 2,250 μg collagen per hr per ml culture filtrate in the assay employed. Since approximately 60 mg dry weight of *B. melaninogenicus* would be required to produce activities in this range, it was estimated that cultures of *C. histolyticum* are more than 100 times as active in hydrolyzing collagen.

The optimal pH for collagenolytic activity was found to be over the range of from pH 6.8 to pH 7.3 (Fig. 3). More acid or alkaline conditions caused a sharp drop in activity. The enzyme was also found to be heat-labile, a temperature of 60 C for 10 min resulting in loss of 65% of activity.

Initial attempts to obtain cell-free preparations of the enzyme using ultrasound, alternating freezing and thawing, and toluene treatment were ineffective. However, exposure to ultrasound in a 9 kc Raytheon sonicator for 15 min did release an enzyme capable of hydrolyzing gelatin and azocoll, but such preparations were devoid of collagenolytic activity. This suggests that the organism may possess more than one

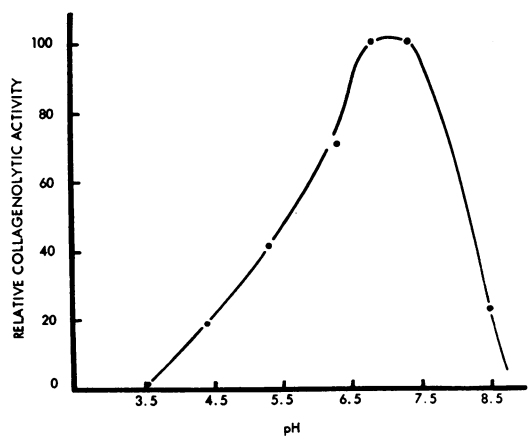

Fig. 3. Effect of pH on hydrolysis of soluble reconstituted collagen by lyophilized cells of *Bacteroides melaninogenicus*.

TABLE 2

Effect of $CaCl_2$ and cysteine on collagenolysis by dialyzed enzyme preparations of Bacteroides melaninogenicus

Dialysis	Reaction Mixture* Plus:	Collagenolytic Activity†
		%
1. Dialyzed 30 hr at 4 C against 0.1 M tris (pH 7.2)	No additive	10
	10^{-3} M $CaCl_2$	7
	10^{-2} M Cysteine	96
	10^{-3} M Cysteine	43
	10^{-2} M Cysteine; 10^{-3} M $CaCl_2$	93
2. Dialyzed 30 hr at 4 C against 0.1 M tris (pH 7.2) containing 0.1% sodium thioglycolate	No additive	25
	10^{-3} M $CaCl_2$	38
	10^{-2} M Cysteine	90
	10^{-3} M Cysteine	81
	10^{-2} M Cysteine, 10^{-3} M $CaCl_2$	100

* Two milliliters of 0.25% soluble collagen, 1 ml cell-free autolysate.

† Per cent release of hydroxyproline compared to activity with added cysteine and $CaCl_2$.

proteolytic enzyme; an observation which in part explains the array of proteinaceous substrates which are attacked.

Cell-free preparations of the enzyme could be obtained by permitting the organisms to autolyze. Autolysates were prepared by incubating fresh cells in either buffer or culture supernatant liquor for 5 days at 37 C under anaerobic conditions. Autolysates were then centrifuged to remove unlysed cells and debris. These preparations contained approximately 50% of the collagenolytic activity possessed by the initial cell suspension. By using concentrated suspensions, autolysates capable of hydrolyzing 1,900 μg collagen per hr per ml autolysate have been prepared. Such autolysates contained from 280 to 350 μg nitrogen per ml after dialysis against distilled water.

Cysteine has been reported to inhibit the collagenase of *C. welchii* (Bidwell, 1950). However, freshly prepared L-cysteine hydrochloride at concentrations of 10^{-2} M or 10^{-3} M did not impair collagenolytic activity of cell-free autolysates of *B. melaninogenicus*; in fact it was slightly stimulatory. Neutralized L-cysteine hydrochloride solutions which were prepared 24 to 48 hr prior to use did exert an inhibitory effect. Presumably the cysteine had become partially oxidized, and thus lost its reducing capacity. The stimulating effect of freshly prepared solutions of L-cysteine hydrochloride was most pronounced when cell-free autolysates dialyzed against 0.1 M tris(hydroxymethyl)aminomethane buffer (tris) at pH 7.2 were employed (Table 2). These preparations were almost devoid of activity unless activated with 10^{-2} M L-cysteine hydrochloride. A comparable effect was observed when the enzyme was dialyzed against buffer containing 0.1% sodium thioglycolate. These data suggest that the enzyme is dependent upon sulfhydryl groups for activity. The observation that mercuric chloride markedly inhibits enzyme activity, and that this inhibition

TABLE 3

Effect of various agents on the hydrolysis of soluble collagen by cell-free autolysates of Bacteroides melaninogenicus

Reaction Mixture* Plus:	Collagenolytic Activity†
	%
No additive	100
10^{-2} M Cysteine prepared 48 hr prior to use	75
10^{-2} M Cysteine	114
10^{-3} M Cysteine	103
10^{-3} M $CaCl_2$	103
10^{-4} M $CaCl_2$	98
5×10^{-2} M EDTA	43
5×10^{-3} M EDTA	46
5×10^{-4} M EDTA	78
5×10^{-3} M EDTA; 5×10^{-3} M $CaCl_2$	88
5×10^{-3} M EDTA; 5×10^{-2} M $CaCl_2$	99
10^{-3} M $HgCl_2$	0
10^{-4} M $HgCl_2$	20
10^{-5} M $HgCl_2$	77
10^{-3} M $HgCl_2$; 10^{-2} M cysteine	38
10^{-4} M $HgCl_2$; 10^{-2} M cysteine	74
10^{-5} M $HgCl_2$; 10^{-2} M cysteine	104
Storage at 4 C for 24 hr	75
Storage at 4 C for 48 hr	35
Storage at 4 C for 24 hr plus 10^{-2} M cysteine	98

* Two milliliters of 0.25% soluble collagen, 1 ml crude cell-free autolysate.

† Per cent release of hydroxyproline compared to control, no additives.

TABLE 4

Effect of heat and natural inhibitors on collagenolysis by lyophilized cells of Bacteroides melaninogenicus

Treatment*	Collagenolytic Activity†
Suspended in 0.1 M tris (pH 7.2)	100
Suspended in 0.1 M tris (pH 7.2), heated 60 C for 10 min	35
Suspended in 0.1 M tris (pH 7.2), heated 70 C for 10 min	0
Suspended in sonic lysate of *B. melaninogenicus*	62
Suspended in sonic lysate of *Escherichia coli*	50
Suspended in human plasma	25

* Thirty milligrams of lyophilized cells in 1.0 ml suspending liquor employed as source of enzyme. Mixed with 2 ml 0.25% soluble collagen.

† Per cent release of hydroxyproline compared to cells in 0.1 M tris buffer (pH 7.2).

is partially reversed by L-cysteine hydrochloride (Table 3) supports this view.

Calcium ions are required for the activity of many proteolytic enzymes, and Gallop, Seifter, and Meilman (1957) have demonstrated such a requirement for clostridial collagenase. The activity of crude cell-free autolysates of *B. melaninogenicus* was not affected by calcium chloride (Table 3), but evidence of a cation requirement was obtained by observing the inhibitory effect of ethylenediaminetetraacetic acid (EDTA). Inclusion of calcium chloride in the reaction mixture completely overcame the EDTA inhibition (Table 3). In repeated experiments calcium ions exerted a slight stimulatory effect on enzyme preparations dialyzed against 0.1 M tris containing 0.1% sodium thioglycolate (Table 2). However, the effect of cysteine was so marked on these preparations that a requirement for calcium ions could not be demonstrated conclusively. It may well be that calcium is tightly bound to the enzyme or to the collagen substrate and is not easily removed by dialysis. Similar difficulties were encountered with clostridial collagenase (Gallop et al., 1957).

Collagenolytic activity of dialyzed enzyme preparations activated with L-cysteine hydrochloride was 2- to 3-fold greater than that of the original crude autolysate. This suggested the presence of an enzyme inhibitor in autolysates which could be removed by dialysis. Such an inhibitor conceivably could be employed by the organism as an enzyme regulator, and for protection of other cell proteins. Additional evidence of naturally occurring enzyme inhibitors was obtained by observing the inhibitory effect human plasma, or sonic lysates of either *B. melaninogenicus* or *Escherichia coli* had on the activity of lyophilized cells (Table 4).

DISCUSSION

B. melaninogenicus is the first oral bacterium which has been shown to hydrolyze native collagen; indeed it appears to be the first asporogenous organism known to possess this ability. The collagenolytic mechanism of the bacterium differs from that of *Clostridium* species. With the latter organisms, collagenase is actively excreted from the cell, whereas the collagenolytic enzyme of *B. melaninogenicus* is associated with the cell.

A second distinction is the amount of collagenolytic activity possessed by cultures of the respective organisms. Cultures of *C. histolyticum* are over 100 times as active as those of *B. melaninogenicus*. Presumably, clostridial cells continually produce and excrete enzyme, whereas cells of *B. melaninogenicus* form only a limited quantity, because it is retained by the cells.

In addition to clostridial collagenase, plant and animal cathepsins have been reported to attack native collagen (Sherry, Troll, and Rosenblum, 1954). However, these enzymes differ from clostridial collagenase in that they are effective only under highly acidic conditions (pH optima of 2.0 to 4.5), and are inhibited by 0.5 M salt solutions. The collagenolytic enzyme of *B. melaninogenicus* appears to be more closely related to the clostridial type, for it is not affected by 0.5 M salt, and functions optimally under neutral conditions. Although cysteine was found stimulatory for the collagenolytic enzyme of *B. melaninogenicus* in contrast to its effect on clostridial collagenase, it is not unusual for enzymes to be activated by sulfhydryl reagents. This is especially so when they are derived from obligately anaerobic organisms.

B. melaninogenicus possesses unique nutritional requirements in that many strains require compounds of the vitamin K group, and virtually all strains require or are stimulated by hemin (Gibbons and Macdonald, 1960). These requirements probably account for the fact that Mer-

genhagen and Scherp (1960) failed to identify any organism responsible for the collagenolytic activity which they demonstrated in mixed cultures from the oral cavity. In mixed culture the organism would be expected to proliferate due to the elaboration of vitamin K compounds by other microorganisms.

Since *B. melaninogenicus* is regularly present in the oral cavity in numbers of 10^8 to 10^9 per g of gingival scrapings (Dale, 1960, *personal communication*), it is tempting to speculate that it may contribute to the pathogenesis of periodontal diseases. The nature of production of the collagenolytic enzyme as well as its release by cell autolysis would help to account for the usual insidious, chronic nature of these conditions. In addition, the organism has been associated with a variety of mixed anaerobic infections (Burdon, 1928; Cohen, 1932; Weiss, 1943; Hite, Locke, and Hesseltine, 1949; Macdonald et al., 1956) in which the collagenolytic enzyme now may be presumed to play a role.

SUMMARY

An oral strain of *Bacteroides melaninogenicus* has been shown to possess an enzyme capable of hydrolyzing native collagen. Gelatin, azocoll, casein, egg albumin, and plasma proteins are also attacked by the bacterium. The collagenolytic enzyme is intimately associated with the cell, but is released during cell autolysis. Quantitative studies were undertaken using soluble collagen as substrate and it was shown that the enzyme functions optimally over a range of pH 6.8 to pH 7.3, and is heat-labile. Calcium ions exerted a slight stimulatory effect on dialyzed enzyme preparations, and overcame ethylenediaminetetraacetic acid inhibition. L-Cysteine hydrochloride activated dialyzed enzyme preparations and also partially reversed inhibition caused by mercuric chloride. A total of 30 strains of *B. melaninogenicus* from the oral cavity and intestinal tract of man and from the rumen of cattle were tested for activity against reconstituted collagen extracted from guinea pig skins. All 30 strains possessed collagenolytic activity against this substrate.

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