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Collagenolytic Activity of Dental Plaque Associated with Periodontal Pathology

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Certain dental plaques, removed from sites of gingival and periodontal pathology in mentally retarded, institutionalized individuals, when incubated in phosphate buffer with Achilles tendon collagen, gave rise to an increase in ninhydrin-positive material. These plaques, while showing great variability, released significantly more ninhydrin-positive material per milligram of plaque (wet weight) than did either the endogenous or heat-treated controls. Certain plaques could also break down soluble, tritiated, labeled collagen isolated from the calvaria of chicken embryos. *Bacteroides melaninogenicus* and *Clostridia histolyticum* were found in plaques by either fluorescent antibody or cultural methods. *C. histolyticum*, when detected, accounted for about 0.01 to 0.1% of the bacteria in plaque. A conspicuous isolate from some plaques was a *Bacillus* species which rapidly liquefied gelatin. Cell-free supernatants of this organism were able to degrade about 50 to 70% of the soluble collagen when incubated at 36 C. *C. histolyticum* ATCC 8034 caused an 80% degradation of the collagen under the same conditions of incubation. The *Bacillus* strains were facultative, could ferment glucose, reduced nitrate to nitrite, and were catalase, indole, and urease negative. The limited taxonomic information for the isolates is compatible with the description given for *Bacillus cereus*.

Periodontal disease is a chronic inflammatory process which results in the loss of the collagen fibers which anchor the tooth to the alveolar bone. Dental plaque bacteria are involved in this tissue destruction. The bacteria do not appear to invade the tissue but rather elaborate a variety of products which are tissue irritants and/or antigens which attract inflammatory cells to the gingival sites (18, 27). The inflammatory response is thought to release collagenase and other enzymes from granulocytes (4, 10), which results in a net loss of tooth-supporting tissue. It is not known whether the inflammatory response is due to the increased bulk of the plaque bacteria, or whether it is due to the colonization of the plaque by an organism(s) which is more antigenic, or produces more irritant or more plaque matrix per unit cell. An association between plaque mass and either periodontal disease (28) or gingivitis (12) can be demonstrated. Collagenolytic organisms have been sought for in dental plaque. Plaque removed from patients with periodontal disease can degrade collagen paper (24), Azocoll (17), and reconstituted collagen (21). The collagenase-producing organisms in these studies were never identified. Subsequently, Gibbons and MacDonald (5) demonstrated that *Bacteroides melaninogenicus*, which comprises about 5% of the cultivable flora from periodontal plaque (6, 15), possesses a collagenase capable of degrading undenatured collagen.

Periodontally associated plaque removed from the tooth surfaces of institutionalized mentally retarded individuals was recently shown to contain *Clostridium histolyticum* and *B. melaninogenicus* (15). The present investigation was initiated to determine whether this plaque would exhibit collagenase activity against undenatured collagen. In the course of the investigation, a facultative *Bacillus* species, which was isolated from high dilutions of certain plaques, was found to exhibit collagenase activity. The experiments which describe the isolation and identification of this organism and which demonstrate its collagenase activity are described.

MATERIALS AND METHODS

Collection of plaque samples. Dental plaque was collected from mentally retarded subjects who were residents of the Plymouth State Home and Training

School, Northville, Mich. Buccal or labial plaque was removed from the cervical area of the teeth whose gingival margin exhibited gingivitis or periodontitis. The degree of the gingivitis was estimated by a modification of the Loe index (11). The plaque was weighed immediately and then, depending upon the experiment, was added to (i) 10 ml of a reduced transport fluid (RTF) (14, 29) for the culturing experiments, (ii) 5 ml of 0.067 M phosphate buffer containing 0.45% NaCl (pH 7.4) for the collagenase assay using insoluble collagen (20), or (iii) 2 or 5 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6) for the collagenase assay using soluble collagen.

Bacteriological procedures. The plaque samples were cultured within 2 to 3 h after collection. The samples were dispersed by ultrasonic sound for 10 s by use of the microtip adapter for the model W1850 sonifier (Heat Systems Ultrasonic Inc.). The plaque was then serially diluted in the RTF, and 50 μliters of appropriate dilutions were inoculated with Eppendorf pipettes onto the surface of MM10 sucrose plates (16). The plates were incubated either anaerobically (85% N_2, 10% H_2, 5% CO_2) or aerobically. After 2 to 7 days of incubation, representative colonies were subcultured, and isolates which were judged to be pure by Gram stain and dark-field examinations were screened for their ability to liquefy gelatin by using thioglycolate gelatin medium (Difco). The isolates which liquefied gelatin within 48 to 72 h were partially characterized by using the following tests: terminal pH in the presence and absence of 0.5% glucose in thioglycolate broth without dextrose (Difco); glucose utilization employing the Glucostat reagent (Worthington Biochemical); nitrate reduction; indole production; and for the presence of catalase and urease (urea broth, Difco). Representative strains were grown in peptone-yeast extract broth with and without glucose (PY and PYG, respectively) (9). After growth occurred, the acid end products, both free and methylated derivatives, were determined by gas liquid chromatographic procedures (9, 13). These procedures had been used previously to speciate bacteria isolated from plaque (15, 16).

Fluorescent antibody procedures. Fluorescent antibody (FA) reagents were prepared against certain species known to possess a collagenase, i.e., *C. histolyticum*, ATCC strain 8034; *C. perfringens*, ATCC strain 13124; *B. melaninogenicus*, strain 94 isolated from gingival plaque of a mentally retarded patient, and *B. melaninogenicus* strains B536 and B684 isolated by Sydney Finegold from human feces and a human wound, respectively. The *C. histolyticum* conjugate gave a 4+ reaction with its homologous vaccine and did not cross-react with vaccines of *C. perfringens* and *C. sporogenes*, an organism known to be present in the plaque smears (14). The various *B. melaninogenicus* conjugates gave a 4+ reaction with their homologous vaccines and a weak 1 cross-reaction with the heterologous vaccines. These weak cross-reactions were considered as negatives when and if they were seen in plaque smears. There were no cross-reactions between the *B. melaninogenicus* antisera and *Bacteroides oralis* strains 7CM and J1. Smears were made of the dispersed plaque and after air-drying were incubated with the working dilution of the conjugated antiserum in a moist chamber at room temperature for 30 min. A 1:20 dilution of aluminum-chelated eriochrome black, which reduces nonspecific staining, was added for 10 to 15 s (7), after which time the slides were rinsed with phosphate-buffered saline (pH 7.6 to 7.8) and distilled water before mounting. The smears were examined with a Zeiss GFL microscope with an Osram 200-W mercury vapor arc lamp and a dark-field condenser. Excitation filter BG12 and barrier filters OG4/GG4 in combination were used. Only cells with 3 or 4+ fluorescence were recorded as positive (22). In some instances the number of fluorescent cells per 25 or 40 high-power microscope fields (hpf) was determined.

Collagenase assay. The ability of plaque suspensions to degrade collagen was measured by two different assays. In one method the release of ninhydrin-positive material from insoluble calf Achilles tendon collagen (Worthington Biochemical) was determined by the method of Mandl et al. (20). In the other method, the release of [^{3}H]proline and [^{3}H]hydroxy proline from soluble collagen obtained from the calvaria of chicken embryos (23) was determined.

The plaque was collected, weighed, added to either the phosphate or Tris buffer, and dispersed by sonic disruption for 10 to 15 s. When the insoluble collagen was used, 0.5-ml samples of the plaque suspensions were added to tubes containing 4.5 ml of 0.067 M phosphate buffer (pH 7.4), 0.45% NaCl, and either 10 or 20 mg of collagen. In other tubes the plaque suspension was heated for 10 min in a boiling-water bath (heat-treated control) or was incubated in the absence of collagen (endogenous control). The addition of $CaCl_2$ to plaque had no effect on the results, presumably due to the high levels of calcium already in the plaque. The collagen was also incubated with one of the following: 0.1 ml of a 0.1% aqueous solution of a crude *C. histolyticum* collagenase (Worthington Biochemical); 100 μg of two-times-crystallized bovine pancreas type III trypsin (Sigma); or alone in the phosphate buffer. The various plaque suspensions were incubated for 72 h at 37 C. Samples were removed at 48 and 72 h and tested for peptide or amino acid release with ninhydrin, using leucine as a standard. In some experiments, the soluble hydroxyproline content was determined by the method of Bergman and Loxeley (1). The suspensions were centrifuged, and 2 ml of the supernatant was hydrolyzed with 2 ml of 12 N HCl in an autoclave for 3 to 6 h. The paradiaminobenzaldehyde used in this assay was a white powder obtained from Matheson, Coleman and Bell. The chloramine T (J. T. Baker) solution was freshly prepared for each determination.

When soluble collagen was used, 1.5 ml of the plaque suspension was added to 0.1 ml of collagen (specific activity 692 counts per min per μg of protein) and incubated at 37 C for 2.5 h or at 35 C for 5 h. The soluble collagen was kept at 4 C in an acetate buffer (pH 4.5) and before usage was dialyzed overnight against 0.05 M Tris-hydrochloride buffer, pH 7.6). Enough $CaCl_2$ to give a final concentration of 0.005 M $CaCl_2$ was added. After incubation, the reaction mixtures were dialyzed overnight against water to remove low-molecular-weight breakdown products. A

0.5-ml amount of the retentate was added to 10 ml of scintillation liquid (Aquasol, Universal L. S. L. Cocktail, New England Nuclear) and counted in a Nuclear-Chicago liquid scintillation counter (model 8731). Controls included the substrate alone, the substrate plus heat-treated plaque, i.e., at 70 C for 30 min, and the substrate plus collagenase, trypsin, or pepsin. In experiments involving isolates of bacteria from the plaque, the cultures were grown overnight aerobically in thioglycolate broth with and without glucose. After centrifugation at 17,000 rpm in a Sorvall RC2B refrigerated centrifuge, 3 or 5 ml of the supernatant was added to 150 μliters of collagen and 0.5 ml of 0.05 M Tris-hydrochloride (pH 7.6). The mixtures were incubated for either 4 h at 36 C or 16 h at 29 C. After dialysis for approximately 16 h against water, 0.5 ml of the retentate was counted in a liquid scintillation counter.

RESULTS

An earlier investigation demonstrated the presence of *Clostridium* species in the plaque of mentally retarded children (15). Additional studies showed that some isolates were capable of aerobic growth, suggesting that they might be strains of *C. histolyticum*. Sixteen such strains were motile, gram-positive rods with oval subterminal spores. They rapidly liquefied gelatin, and did not lower the pH in PYG broth. Only acetic acid was produced in PYG broth. All strains were catalase negative, failed to produce indole, and did not reduce nitrate. Strains cross-reacted with a fluorescent antibody to *C. histolyticum* ATCC strain 8034. Also, some plaque smears showed the presence of rods which gave a 3 or 4+ reaction with the strain 8034 conjugate. The number of FA-positive cells in the smears was low, usually being less than 1% of the total cells.

Studies were then performed to determine whether samples of plaque would exhibit any collagenase activity. Certain plaques, when incubated with Achilles tendon collagen, apparently hydrolyzed this substrate as judged by an increase in ninhydrin-positive material (Table 1). As the plaque bacteria were incubated in the absence of nutrients, this increase could be due to degradation of the collagen, to endogenous catabolism of either plaque matrix protein or bacterial protein, or to a combination of both. These possibilities were resolved by comparing the collagen-containing tubes with the endogenous controls and heat-treated controls. Thirty-two plaque samples taken from subjects with various degrees of gingivitis released approximately 86 ± 50-μg leucine equivalents per mg of plaque wet weight from the collagen-containing mixtures. These plaques, while showing great variability, released significantly more ninhydrin-positive material per milligram of wet weight than either the endogenous or heat-treated controls, i.e., $P < 0.01$ (student t test) (Table 1). The experiments were interpreted as showing that plaque suspensions were capable of releasing small amounts of ninhydrin-positive material from insoluble collagen. Smears from several plaques were stained with FA conjugates against *C. histolyticum*, *C. perfringens* and the *B. melaninogenicus* strains. All six subjects had either *C. histolyticum* or *B. melaninogenicus* in their plaque and three subjects had both species (Table 2). Only the oral isolate of *B. melaninogenicus* stained the plaque cells, whereas the fecal strains were not detected. Five of these plaques released ninhydrin-positive compounds from the collagen after correction for endogenous catabolism (Table 2). Three of five ninhydrin-positive plaques released variable amounts of hydroxyproline into the buffer.

TABLE 1. *Release of ninhydrin-positive compounds from insoluble collagen by suspensions of dental plaque*[a]

Collagen	Enzyme	Leucine equivalents (μg) released	Leucine equivalents (μg)/mg of plaque (wet weight)
20 mg	Collagenase (100 μg)	1,654 ± 232[b] (6)[c]	
20 mg	Trypsin (100 μg)	152 ± 45 (3)	
20 mg	Blank	92 ± 60 (6)	
20 mg	Plaque (2.7 mg wet wt)[d]	232 ± 164 (32)	86.1 ± 50.2[e]
	Plaque heated	100 ± 86 (26)	37.3 ± 26.4
	Plaque	103 ± 80 (31)	38.2 ± 22.2

[a] Reaction mixture included 0.5 ml of enzyme or plaque suspension in 0.067 M PO_4 buffer (pH 7.4), 4.5 ml of 0.067 M PO_4 buffer (pH 7.4), 0.45% NaCl with and without 20 mg of Achilles tendon collagen, incubated for 48 h at 37 C.

[b] Average ± standard deviation – enzyme values, corrected for heat-inactivated enzyme control.

[c] Number in parentheses indicates number of trials or number of plaque samples.

[d] Average weight of 32 separate plaque samples.

[e] The values for plaque plus collagen are significantly higher than the values obtained from the heated plaque or plaque.

The ability of certain plaque suspensions to degrade collagen was also tested using a soluble ^{3}H-labeled collagen (23). This collagen was degraded by the crude collagenase obtained from *C. histolyticum*, but was resistant to hydrolysis by trypsin, pepsin, and heat-inactivated plaque (Table 3). Plaques were removed from periodontally involved sites on several occasions. The data from two such series are shown in Table 3. In series 1, all five samples caused a reduction in counts when compared to the collagen blank, and the values ranged from 535 to 3,699 counts per min per mg of plaque

TABLE 2. *Detection of collagenase-producing bacteria in dental plaque by fluorescent antibody and the ability of plaque to release amino acids from insoluble collagen*[a]

Conjugate	Dilution	Bacteria detected in plaque by FA methods					
		1[b]	2	3	4	5	6
C. histolyticum ATCC 8034	1:2	+	−	+	+	+	+
C. perfringens ATCC 13124	Undiluted	−	−	−	−	−	−
B. melaninogenicus Oral-94	1:8	+	+	−	+	+	−
Fecal B536	1:8	−	−	−	−	−	−
Wound B684	1:4	−	−	−	−	−	−
Amino acid (μg) released per mg of plaque[c]		18.1	16.3	72.6	24.3	54.9	0
Hydroxyproline (μg) released per mg of plaque		0	2.5	26.4	9.8	0	0

[a] Incubation mixture contained 0.5 ml of plaque suspension, 4.5 ml of 0.067 M PO_4 buffer (pH 7.4), 0.45% NaCl, and 20 mg of Achilles tendon collagen incubated for 48 h at 37 C.
[b] Sample number.
[c] Values are corrected for endogenous controls.

TABLE 3. *Release of radioactivity from ^{3}H-labeled soluble collagen by suspensions of dental plaque*[a]

Subject	Enzyme source	Activity in the retentate (counts/min)	Reduction from blank or heat-inactivated plaque (%)	Loss of activity per mg of plaque (counts/min)	*C. histolyticum* on FA smear[b]
Series 1					
	Blank	21,708[c]			
	Collagenase (100 μg)	2,867	87		
	Trypsin				
	100 μg	21,806	0		
	1,000 μg	22,806	0		
	Pepsin (500 μg)	22,508	0		
B.C.	Plaque (1.7 mg)	20,689	5	600	−
D.S.	Plaque (6.3 mg)	18,343	16	535	+
D.S. #2	Plaque (8.2 mg)	17,650	19	495	+
T.O.	Plaque (2.1 mg)	13,939	36	3,699	−
R.M.	Plaque (5.7 mg)	16,136	26	977	−
Series 2					
V.S.	Heat inactivated plaque[d]	23,406	0		+
E.G.	Heat inactivated plaque[d]	23,446	0		−
D.D.	Heat inactivated plaque[d]	25,058	0		−
V.S.	Plaque (1.4 mg)	21,310	11	1,900	+
E.G.	Plaque (3.2 mg)	20,046	16	1,226	−
D.D.	Plaque (2.3 mg)	19,882	17	1,777	−
P.C.	Plaque (3.2 mg)	25,030	0		−
D.S. #3	Plaque (2.8 mg)	25,324	0		+
T.O. #2	Plaque (2.0 mg)	20,864	13	1,553	−
R.M. #2	Plaque (3.2 mg)	24,080	0		+
C.C.	Plaque (2.0 mg)	20,037	16	1,970	+
K.J.	Plaque (1.7 mg)	23,076	4	525	−

[a] Reaction mixture included 1.5 ml of plaque suspension, 0.5 ml of 0.05 M Tris-hydrochloride (pH 7.6), 0.1 ml of collagen, 0.005 M $CaCl_2$, incubation at 37 C for 2.5 to 5 h.
[b] Presence or absence on plaque smear stained with *C. histolyticum* conjugate.
[c] Average of duplicate determinations.
[d] Plaque heated at 100 C for 10 min. Average of three heat-inactivated plaques, 23,970 counts/min.

(wet weight). In series 2 six of nine plaque samples exhibited a reduction in counts when compared to heat-inactivated plaque (range 0 to 1,970 counts per min per mg of plaque [wet weight]). Collagenase activity was detected in some cases from only one of the two separate sites sampled in the same individual. Concurrent cultural studies were unable to demonstrate *C. histolyticum* on high-dilution plates, but this organism was detected in low numbers, i.e., 0.01 to 0.1% of the cells in 7 of 17 plaques by FA examination (Table 3).

A conspicuous isolate on the high-dilution plates was a motile, thick, gram-positive rod which was found on both the aerobically and anaerobically incubated plates. All isolates liquefied gelatin within 24 to 48 h. Representative strains were grown overnight and centrifuged, and the supernatant was added to the soluble collagen. Three strains, i.e., strains 1, 3, and 10, possessed an extracellular enzyme which was capable of degrading the soluble collagen by 50 to 70% when compared to the heat-treated controls (Table 4). Other plaque isolates, such as two motile gram-positive rods capable of liquefying gelatin, i.e., strains 5 and 12, as well as a peptostreptococcal strain, were unable to degrade the collagen (Table 4). *C. histolyticum*, strain ATCC 8034, caused a 77% reduction in the radioactivity of the soluble collagen. Strain 3, which hydrolyzed the collagen to the greatest extent (i.e., 72%), was studied further. Cells were grown overnight in thioglycolate broth plus 0.5% glucose in the presence or absence of the Achilles tendon collagen. The cells were harvested as before and 3 or 5 ml of the supernatant was incubated with the soluble collagen for 16 h at 29 C, so as to minimize any heat denaturation of the collagen. Under these conditions the *C. histolyticum* collagenase caused a 63% reduction in counts (Table 5). Three and five milliliters of the supernatant reduced the counts by 19 and 28%, respectively, when grown in the absence of collagen, and by 23 and 37% when grown in the presence of collagen.

The 3 collagenase-producing strains and 16 similar isolates were partially characterized and identified as a facultative *Bacillus* species. These strains grew more luxuriantly aerobically than anaerobically. They utilized glucose, lowering the pH to about 5.2 to 5.8. However, good growth was obtained in thioglycolate medium without dextrose and in a chemically defined tissue culture medium 199 (Gibco). Lactate, acetate, and succinate were formed anaerobically in the thioglycolate medium both in the presence and absence of glucose. In medium 199, only lactate and acetate were formed. Motile cells were common in overnight growth, but rare in older cultures. Occasionally a spore was noted in the cells. Four representative strains were inoculated into thioglycolate broth with 0.5% glucose and then the broth was heated in a water bath at 100 C for 10, 30, and 60 min. All cultures heat treated for 10 and 30 min and three of four treated for 60 min grew upon subsequent incubation at 37 C. All strains were able to reduce nitrate to nitrite, did not ferment mannitol, possessed a catalase, lacked a urease, and were indole negative. Upon microscope examination, they appeared as thick, granular rods with blunt ends, sometimes in chains, which were about 0.8 to 1.2 μm thick and 3 to 6 μm long. Colonial growth on aerobically incubated MM10 sucrose agar consisted of gray-white to cream, soft, moist, swarming colonies which exhibited a beta hemolysis. After several days at room temperature the older growth would acquire a tan pigmentation which contrasted with the gray-white color of the spreading margins.

TABLE 4. *Degradation of ^{3}H-labeled soluble collagen by cell-free supernatants of bacteria isolated from dental plaque*[a]

Bacteria	Heat treatment 70 C for 30 min	Radioactivity in retentate (counts/min)	Reduction in activity (%)
Bacillus sp.			
Strain 1	+	19,904	50
	−	9,937	
Strain 3	+	19,134	72
	−	5,445	
Strain 10	+	19,342	61
	−	7,623	
Motile gram-positive rod			
Strain 5	+	21,661	7
	−	20,137	
Strain 12	+	22,046	1
	−	21,850	
Peptostreptococcus sp. Strain 16	+	23,100	0
	−	23,428	
Clostridium histolyticum ATCC 8034	+	18,186	77
	−	4,094	

[a] Reaction mixture includes 5 ml of supernatant, 0.5 ml of 0.05 M Tris-hydrochloride (pH 7.6) containing 0.005 M $CaCl_2$, and 150 μliters of collagen; incubated for 4 h at 36 C with mixing every 30 min. The enzyme source for all strains was 5 ml of supernatant.

TABLE 5. *Degradation of soluble ^{3}H-collagen by cell-free supernatants of Bacillus sp. strain 3*[a]

Test	Enzyme source	Heat treatment (70 C for 30 min)	Radioactivity in retentate (counts/min)	Reduction in count (%)
Control[b]	Blank	–	23,149	63
	Collagenase	–	8,600	
Experimental	Cells grown in absence of collagen			
	5 ml of supernatant	+	21,746	
	3 ml of supernatant	–	17,510	19
	5 ml of supernatant	–	15,580	28
	Cells grown in presence of insoluble collagen			
	5 ml of supernatant	+	22,354	
	3 ml of supernatant	–	17,123	23
	5 ml of supernatant	–	14,057	37

[a] Reaction mixture included supernatant, 0.5 ml of 0.05 M Tris-hydrochloride (pH 7.6), 150 μliters of collagen, 0.005 M $CaCl_2$; incubated for 16 h at 29 C.

[b] A 5-ml amount of supernatant heated at 70 C for 30 min, and then used as blank or collagenase added.

DISCUSSION

Dental plaque removed from sites of gingival and periodontal pathology in mentally retarded institutionalized individuals contained at least three distinct microbial species which appear capable of degrading undenatured collagen. *B. melaninogenicus* and *C. histolyticum* were previously found by cultural procedures (15) and in this investigation were demonstrated by FA techniques to be present in certain plaques. However, these two organisms were present in low numbers or not detected in plaques which exhibited an ability to degrade soluble collagen. Cultural studies revealed that these plaques contained a motile, sporulating, gram-positive, facultative rod. This organism appeared to produce a soluble collagenase and because of its presence in high numbers in the plaque would seem to be responsible for the collagenolytic activity of the plaque. The precise identity of this organism is not known, but, as an aerobic sporeformer, it most likely is a *Bacillus* species. *Bacillus* species are not thought to possess a collagenase (26). However, *B. cereus* and *B. anthracis* will degrade the collagen in decalcified bone (3), and Weinberg and Randin (30) reported that an aerobe *B. anthracoides*, i.e., the pseudoanthrax bacillus (31), would digest small pieces of fresh Achilles tendon. MacLennan et al. (19) surveyed several genera for collagenase activity and found *B. cereus* and *B. mesentericus* to lack a true collagenase, but to be capable of degrading Azocoll. Their strains of *B. anthracis* did not degrade either collagen or Azocoll. The limited taxonomic information for our isolates is compatible with the description given for *B. cereus* (31). Additional studies will be necessary to determine exact taxonomic status and to reexamine the question as to whether known *B. cereus* isolates contain a collagenase.

The isolation of collagenase-producing organisms from plaque does not mean that collagenase is produced in vivo. This would require the demonstration of collagenase in plaque incubated with collagen under conditions of no growth, i.e., a resting cell suspension where there are no exogenous nutrients. In the present experiments, resting cell suspensions of certain plaque samples consistently released significantly more ninhydrin-positive material from insoluble collagen than did endogenous or heat-treated controls. However, there were always some plaques which failed to do so.

The measurement of collagen degradation by an increase in ninhydrin-positive material has the complication that the plaque organisms could metabolize the released peptides or amino acids, including hydroxyproline (21). This phenomenon could account for the absence of hydroxyproline in some samples in which collagen degradation occurred (Table 2). The overall breakdown of the insoluble collagen as judged by the ninhydrin method was only 0.5 to 1% after 48 h of incubation. This amount of ninhydrin-positive material was comparable to the amount released from the collagen by 100 μg of trypsin (Table 1). This could mean that the plaques either contained the proteolytic equivalents of 100 μg of trypsin per 2.7 mg (wet weight) or much smaller amounts of an enzyme which had collagenolytic activity. The latter possibility was thought to be more probable. Plaque is a complex mixture of bacteria, glycoproteins, polysaccharides, inorganic salts, and 80% water (8,

14). Nitrogen accounts for about 10% of the dry weight (14). If the nitrogen content is converted to protein by multiplying by 6.25, then the present plaque samples contained about 340 μg of protein. Most if not all of this plaque protein and nitrogen content can be accounted for by the 7×10^8 to 11×10^8 bacteria found in 1 mg of plaque dry weight (14) and by the salivary glycoproteins which form the plaque matrix. Any enzymes present in the plaque would make a minimal contribution to the total protein content and certainly would not amount to about 100 μg of trypsin-like proteolytic activity. Thus the data from the incubation of plaque with insoluble collagen seemed to indicate a very low content of collagenase activity in some of the plaque samples. Therefore, a more sensitive assay using a soluble ^{3}H-labeled collagen was employed. This showed that 10 of 15 plaque samples were capable of hydrolyzing this substrate to a varying degree, i.e., 4 to 36%. The results of the collagenase assay with both insoluble and soluble collagen demonstrate that plaque removed from some institutionalized subjects possessed collagenolytic activity.

Mentally retarded institutionalized individuals develop periodontal disease at an early age (15). This situation is generally attributed to the inability of these individuals to brush their own teeth. Cutress, in a comprehensive study (2), compared oral hygiene and other parameters in institutionalized and noninstitutionalized mentally retarded subjects. He interpreted his data as indicating that unknown environmental factor(s) played an important role in the severity of the disease in the institutionalized population. One possible environmental factor could be the colonization of the dental plaque by an organism(s) with pathogenic potential for the gingival and periodontal tissues. Organisms capable of producing collagenase have been suspected of being of etiologic significance in periodontal disease (5, 18). However, with the exception of *B. melaninogenicus*, these organisms have not been isolated from dental plaque. Thus the demonstration of *C. histolyticum*, the *Bacillus* sp., and *B. melaninogenicus* in plaques removed from periodontally involved teeth offers the possibility that these collagenase-producing organisms may be contributing to this disease. Also some attention should be given to determine whether the *Bacillus* sp. as well as *C. histolyticum* are found only in dental plaques of individuals resident at the Plymouth State Home and Training School or if they have a wider oral distribution, particularly in individuals with access to soil and fecal contamination. In this regard, *C. histolyticum* collagenase has been demonstrated by FA methods in the plaque of Guatemalan Maya Indians (R. Morhart, personal communication) whose clinical periodontal conditions resemble that of the institutionalized mentally retarded.

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