Specific Collagenase From Germ-Free Rat Gingivae (35069)

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(Introduced by R. O. Greep)

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A collagenase from a vertebrate source was first described from cultured tadpole tails by Gross and Lapiere (1), and Lapiere and Gross (2). Soon thereafter, collagenase was detected in culture fluids of gingivae from man (3–8), and from the guinea pig (8). The specific collagenase was subsequently detected in culture fluids of human epithelial and connective tissue gingival fragments cultured separately (9). The present investigation discloses the presence of a specific collagenase in culture fluids of gingivae from germ-free as well as conventional rats. This study provides additional proof of a tissue source of collagenase.

Materials and Methods. Free and attached gingivae surrounding molar and incisor teeth of 30-day-old Sprague–Dawley rats (six germ-free and six conventional) were excised, diced into 2- to 3-mm^3 pieces, cleansed in several changes of Tyrode's solution with antibiotics and cultured in either (1) Tyrode's solution with antibiotics on the surface of reconstituted collagen gels, or (2) Tyrode's solution with antibiotics as described previously (3, 9). All were cultured in 5% CO_2 in air at 37° for 7 days. Cultures on collagen gels were not disturbed for the 7-day period. Culture fluids from the others were withdrawn and replenished daily.

Reconstituted collagen was prepared from rat skins according to the method of Kang *et al.* (10). Purity and native helicity of the collagen was determined by optical rotation and resistance to tryptic digestion. Reaction mixtures containing 0.5 μ trypsin per microgram collagen never exceeded 5% digestion.

Collagenase in the pooled culture fluids was concentrated by ammonium sulfate precipitation, passed through a Millipore filter and dialyzed against 0.05 *M* Tris buffer pH 7.9 containing 0.3 *M* $CaCl_2$ as previously described (9). The enzyme is in the 60% saturated ammonium sulfate precipitate after discard of the 20% saturated ammonium sulfate precipitate.

Very little enzyme from culture fluids of germ-free rat gingivae was detected on initial viscometric assays. For this reason incubations were prolonged for 3 days. Reaction mixtures containing 3.0 ml collagen (2 mg/ml 0.1 *M* $CaCl_2$ in 0.05 *M* Tris buffer pH 7.9), 13.0 ml enzyme concentrated by ammonium sulfate precipitation described above, 2.0 ml buffer (0.05 *M* Tris pH 7.9 containing 0.3 *M* $CaCl_2$) and antibiotics (pencillin 100 μg/ml, Mycostatin 100 μg/ml and streptomycin 100 μg/ml) were incubated at 25° for 3 days. Reaction products were precipitated at 4° after addition of sufficient NaCl to make a 20% solution, and the supernatant fluid discarded after centrifugation at 48,000*g* for 45 min. In preparation for acrylamide gel electrophoresis, the collagen was denatured with 8 *M* urea adjusted to pH 5.3 with 0.1 *M* HCl, and dialyzed overnight against the same acidified urea solution. Acrylamide gel electrophoresis was conducted according to the method of Sakai and Gross (11).

Results. Collagenolytic activity derived from cultures of germ-free rat gingivae was demonstrated by two methods. Native collagen gels were digested in the vicinity of cultured germ-free rat gingivae (Fig. 1). Secondly, fragments of α and β polypeptide chains as well as full length α and β chains of collagen were disclosed on acrylamide gels. The patterns were derived from electrophoresis of reaction mixtures obtained after incu-

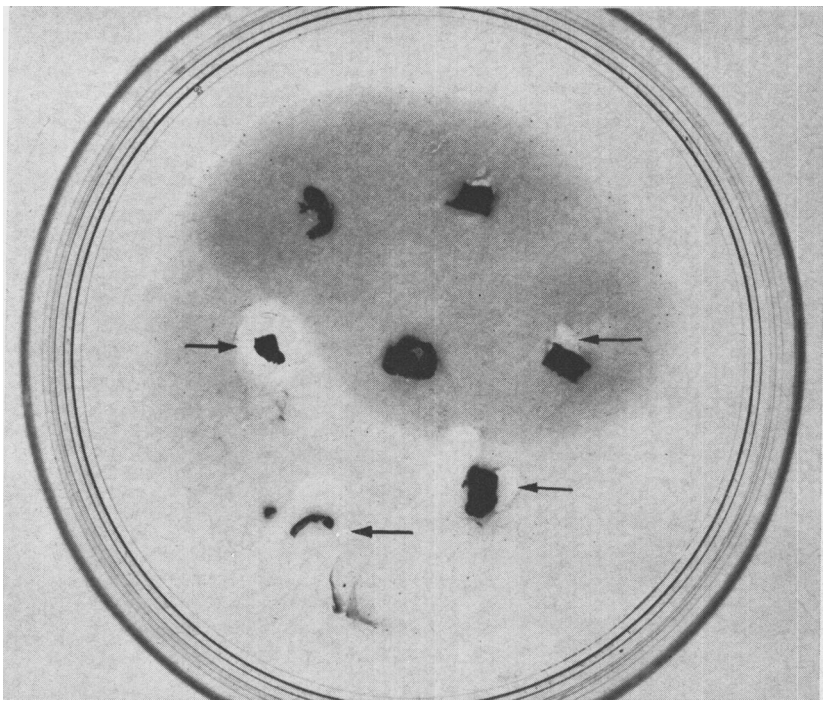

FIG. 1. Collagen gel plate on which germ-free rat gingivae (dark objects) were incubated in Tyrode's solution with antibiotics for 7 days. Note lysis of collagen gel in the vicinity of several of the gingivae (arrows).

bation of collagen solutions with ammonium sulfate concentrated enzyme from culture fluids of germ-free rat gingivae (Fig. 2). Likewise, cultures of gingivae from conventional rats displayed lysis of collagen gels and digestion of collagen molecules in a manner identical to that portrayed in Figs. 1 and 2.

Discussion. Gingivae are always laden with multitudes of microorganisms. Certain kinds of microorganisms commonly associated with gingivae of man are known to produce collagenase; for example, *Bacteroides melanogenicus* (12). For this reason, it is essential to discern if the collagenase detected after gingival culture is derived from the gingival tissues or the associated microorganisms. Utilization of germ-free rat gingivae represents one method of resolving this question.

The experiments described herein unequivocally demonstrate collagenase activity derived from germ-free as well as conventional rat gingivae inasmuch as (a) the control acrylamide gels exhibited typical α and β chains of denatured collagen (Fig. 2), and (b) acrylamide gel electrophoresis patterns of the experimental reaction mixtures after collagenase activity revealed collagen fragments as well as typical α and β chains of collagen (Fig. 2). Thus, specific collagenase activity derived from culture fluids of germ-free and conventional rat gingivae has been demonstrated.

Collagenase derived from man and tadpole tissues act only once on the collagen molecule to form approximately ¼ and ¾ pieces (10, 11, 13–21). On the basis of the experiments conducted for this report, one cannot determine where the rat collagenase acts on the collagen molecule. Some fragments traveled near the marker dye, and for this reason are believed to be relatively small. Whether the small fragments are the result of specific collagenase action is unknown. The enzyme preparation used was crude, and probably contained many enzymes other than collagenase. Purified enyzme preparations are

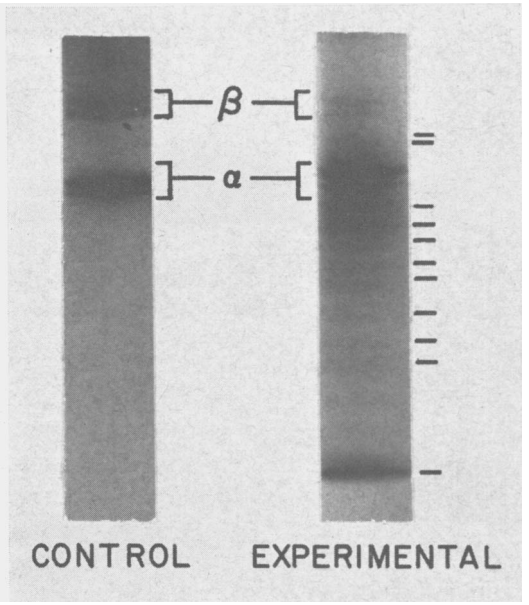

FIG. 2. Acrylamide gel patterns of undigested collagen (control) denatured after the experiment was conducted, and collagen digested by enzyme from germ-free rat gingivae (experimental) and subsequently denatured.

required to answer this question. In order to investigate this problem, collagenase derived from human gingivae and bones is being isolated and purified in this laboratory.

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