Lysis by Human Oral Bacteria of Collagen Altered By Ethylene Oxide. (25468)

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(Introduced by Isadore Zipkin)
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Sterilization with ethylene oxide increased digestibility of collagen in tissue by broth cultures inoculated with human gingival accumulations(1,2,3). Failure of digestion by several pure cultures isolated from such mixtures indicated a synergistic mechanism of collagenolysis(1). Similarly, collagen fibrils reconstituted from acid-soluble collagen of rabbit skin were digested by such mixed cultures but not by pure isolates from them, either alone or in combination(4). However, after treatment with ethylene oxide the reconstituted collagen was digested moderately by a few of these same pure cultures and extensively by their combination. Concomitant morphological alterations of collagen fibrils were followed by electron microscopy.

Materials and methods. The test for collagenolysis was similar to that used previously (4). Two-ml aliquots of a neutral 0.2-0.5% solution of acid-soluble collagen were gelled at 37°C and exposed to 11% ethylene oxide gas in a desiccator for 16 hours. Eight ml of a special hydroxyproline-free broth were added and inoculated with 0.1 ml portions of 48-hour broth cultures of the respective pure cultures. The tubes were then incubated for 7 days at 37°C under 95% nitrogen and 5% CO_2. Following centrifugation, aliquots of sediments and supernatants were analyzed for hydroxyproline after acid hydrolysis. Since the oral microbiota readily catabolizes the hydroxyproline-containing products of collagenolysis, the loss of total hydroxyproline served as an indicator of extent of collagenolysis(4). Five strains of Gram-negative fusiform bacilli (F1, F3, F4, F5, F7) were isolated from human saliva on a selective medium(5) and transferred to fluid thioglycollate medium (Difco) containing 0.5% calcium carbonate. Five strains (V1, V2, V3, V4, V5) of Gram-negative anaerobic cocci (genus *Veillonella*) were isolated from human saliva on a selective medium(6) and maintained in the same medium lacking agar, vancomycin, and basic fuchsin. Two strains of diphtheroid bacilli (PC7, PC8) and 3 strains of viridans streptococci (PC1, PC2, PC6) were isolated on sheep-blood agar from cultures of mixed human gingival scrapings in a casein hydrolyzate broth(4) and subcultured in this broth with addition of 0.5% glucose. Specimens of the reconstituted collagen were prepared for electron microscopy in the form of dried microdrops of suspensions and as thin sections of gel. For the former, small quantities of gel were rendered salt free by repeated suspension in triple-distilled water and centrifugation. Microdrops of final suspensions were placed on electron microscope screens covered with carbon substrates, allowed to dry in air, and shadowed with palladium. Gel samples to be sectioned were fixed for 2 hours in a buffered osmium tetroxide-potassium dichromate mixture, dehydrated in ethanol, and embedded in a mixture of butyl and methyl methacrylates. Sections were cut with a Porter-Blum microtome equipped with glass knives. To afford closer study of the fibrils, the embedding medium was removed from some of the sections, which were then shadowed with palladium.

Results. Marked alterations in morphological characteristics of the gels after ethylene oxide treatment were observed under the electron microscope in both microdrop and sectioned preparations. Before treatment the gels consisted almost entirely of a meshwork of typical collagen fibrils with clearly defined 640A cross-striations (Figs. 1 and 4). After long exposures (16 hours) to ethylene oxide only a few of these fibrils could be found, and the gel seemed instead to be made up of much finer unstriated fibrils, arranged either as a loose network (Fig. 2) or as a compact mass (Fig. 3). Concomitantly, the collagen tended to pass from gel to sol state. Since, however, all hydroxyproline-containing material remained completely precipitable by 2.5% tan-

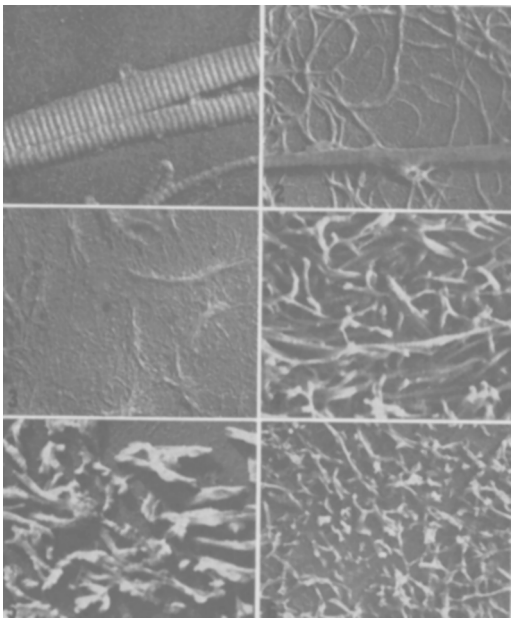

FIG. 1-3. Electron micrographs of microdrop suspensions of heat-gelled acid-soluble collagen before (Fig. 1) and after (Fig. 2, 3) exposure to 11% ethylene oxide gas for 16 hr. × 15,000.

FIG. 4-6. Electron micrographs of sections of heat-gelled acid soluble collagen before (Fig. 4) and after exposure to 0.1% aqueous ethylene oxide for 1 hr (Fig. 5) and 3 hr (Fig. 6). × 11,500.

nic acid, the collagen molecule itself was presumably not split. When a series of specimens exposed for shorter periods was examined to determine the way in which the collagen fibrils disintegrate, an impression was gained that they first become somewhat swollen, then break up into short lengths and finally decompose completely into the fine fibrillar units. Pronounced alterations were observed after as little time as one hour (Fig. 5) and by the end of 3 hours most fibrils completely lost their identity as collagen (Fig. 6). Fibrillar swelling similar to that seen in Fig. 5 was reported in histological sections of human gingival tissue after exposure to ethylene oxide vapor(2).

Table I presents the essential results of a typical experiment demonstrating that these altered collagen fibrils became slightly susceptible to digestion by certain pure cultures of oral bacteria, but most significant was the observation that in combination these cultures effected a 77% reduction in the collagen present as compared with a 59% reduction by *Clostridium histolyticum*. In repeated tests these cultures and their combination failed completely to attack untreated reconstituted collagen(4). These results substantiate previous suggestions of a synergistic mechanism of bacterial lysis of altered collagen(1,3). However, since growth of such organisms as the fusiform bacilli was generally greatly enhanced in the combined cultures, the increased digestion might have resulted at least in part from symbiotic stimulation of growth and metabolism. Finally, it is certainly possible that other combinations of oral bacteria might effect similar reactions.

The microscopic changes observed in these altered collagen fibrils recall the electron microscopic demonstration that collagen fibrils, in areas of rabbit skin where inflammation had been induced by the Arthus phenomenon, exhibited an absence of normal periodic cross striations, variations in density, and irregularities of the margins with evidence of swelling and fragmentation(7). Similarly, the increased susceptibility to bacterial degradation of the collagen fibrils shortened by ethylene oxide (Fig. 6) recalls the report(8) that when native tendon was cut into small pieces (less than 1 mm), the fibers became digestible by trypsin, indicating that they were digested primarily at the cut ends (*cf.* also (9)). In other words, finely divided collagen should be more readily attacked by enzymes. Collagen fibrils in areas of inflammation, like those altered by ethylene oxide, might accordingly be considerably more susceptible to enzymatic digestion, *e.g.*, by local tissue cathepsins(10). This could mean also that the breakdown and

TABLE I. Digestion of Ethylene-Oxide-Treated Reconstituted Collagen by Single and Combined Pure Cultures of Oral Bacteria.

Cultures tested (strains)	% loss in total hydroxyproline
None	0
Diphtheroid bacilli (PC7, PC8)	20, 0
Fusiform " (F1, F3, F4, F5, F7)	0, 0, 16, 18, 0
Streptococci (PC1, PC2, PC6)	0, 0, 0
Veillonellae (V1, V2, V3, V4, V5)	0, 18, 18, 18, 0
Above cultures combined	77, 77
C. histolyticum	59

loss of collagenous fibers, as seen for example in periodontitis, results from primary alteration by the inflammatory response and secondary attack by bacterial proteases.

Summary. Treatment with ethylene oxide rendered reconstituted acid-soluble collagen readily digestible by combined pure cultures of human oral strains of fusiform bacilli, diphtheroid bacilli, streptococci, and veillonellae. The fact that only a few of these strains individually attacked such altered collagen to a slight extent indicates digestion by synergistic and symbiotic reactions between members of the oral microbiota. Concomitantly with increased digestibility from treatment with ethylene oxide, reconstituted collagen exhibited marked morphological changes as revealed by electron microscopy.

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Separation of 2 Glutamic-Oxalacetic Transaminases by Paper Electrophoresis.* (25469)

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The enzyme glutamic-oxalacetic transaminase (GOT) has attracted considerable interest recently because its concentrations in blood and tissue are altered characteristically in a number of disease states. Methods of purification devised up to now(1,2) have produced no indication that this enzyme may occur in more than one form. We observed that crude GOT in an extract of cardiac muscle from dog, when chromatographed on certain ion exchangers, behaves as a mixture of 2 enzymes. When CM-cellulose was employed with phosphate buffer of pH 7.0 and $\Gamma/2 =$ 0.01 or less, one component was adsorbed and could be eluted at higher ionic strength and lower pH, while the other was adsorbed very little if at all. With DEAE-cellulose the behavior of the 2 fractions was reversed. These findings suggested that the 2 GOT's may have opposite electrical charges at pH 7. To test this assumption, the paper electrophoresis of crude preparations of GOT was investigated.

Methods and materials. Tissues were minced and extracted in a blendor with 3 volumes of cold water. The centrifuged extracts were concentrated several fold by ultrafiltration at 0°C. The solutions were recentrifuged at 30,000 g. They were then applied, in proportions of 10 to 40 μl, containing about 1 to 5 mg of protein, to the paper strips in a Spinco electrophoretic apparatus Model R. Phosphate buffer of pH 7.4, $\Gamma/2 = 0.075$, was used, often with the addition of one per cent bovine serum albumin as a stabilizer of enzymatic activity. Stabilization could be achieved also by applying quantities of tissue extracts corresponding to the larger amount of protein. Current was 5 milliamperes, temperature 8°C,

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