against attaining a high concentration in the skin. Topical application would also appear to be an unreliable approach to the epidermis especially in the case of human skin lesions since the conditions of interest present an unusually thick keratin barrier. Furthermore, the absence of sebaceous glands in plantar skin would almost preclude penetration of vit. A at this most common site of verrucae and other hyperkeratoses since the sebaceous glands form the normal pathway for absorption of the vitamin. Intraepidermal injection of vit. A is, of course, a practical impossibility, so that, intracutaneous injection would appear to be the only efficient method of introducing vit. A at the site of an epidermal lesion.

There seems to be only one possible interpretation for the results here reported. The presence of large (hence young appearing) nuclei in the basal and spinous cell layers of the epidermis coupled with a normal rate of mitosis, indicates that the physiologic sequence of maturation and death of these cells has been slowed. Therefore, cells of all types have accumulated and less keratin has been laid down. If these changes occur also in human skin it is reasonable to suppose that injection of vit. A at the site of an heloma would reverse the pathologic process since this lesion shows a central area of atrophy of the epidermis with an overlying plug of horny material. The mechanism of action in the case of verruca, a proliferative virus disease, is still obscure and future work will be directed toward its clarification.

Summary. It has been found that a single intracutaneous injection of vit. A in the rabbit causes an increase in thickness of the epidermis (excluding corneum) which is due to an increase in both number and size of the cells in the germinative and granular layers. The corneum becomes thinner and denser. The occurrence of large, young appearing nuclei in the absence of an increase in mitotic rate indicates that the maturation process is slowed.

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Resistance of Human Gingival Collagen to Human Gingival Bacteria.* (23421)

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Since breakdown of the collagen fibers that anchor the teeth is a principal feature of periodontal disease, the demonstration that microorganisms present in the gingival sulcus or periodontal pocket produce collagenases assumes importance. Investigators using collagen paper or azocoll as substrates(1,2) have reported isolation of oral and gingival organisms that exhibited either collagenase-like or true collagenase activity. However, other proteolytic enzymes, such as trypsin, are capable

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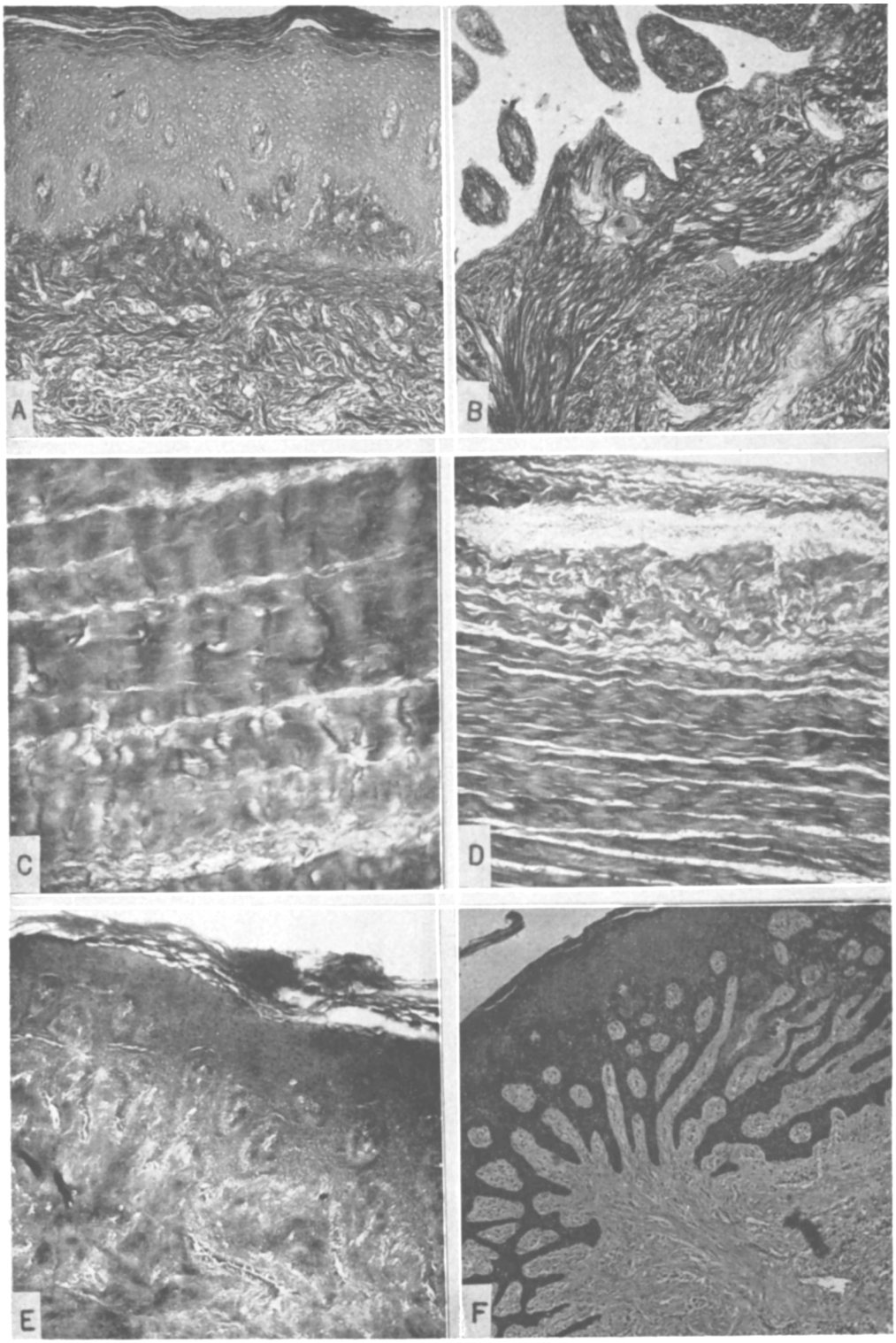

FIG. 1. (A) Section of human gingiva incubated under sterile conditions in broth for 10 days. Cells of stratum granulosum and stratum spinosum are normal, as also is the dense collagenous

of degrading both collagen paper and azocoll, *i.e.*, denatured collagen. In an attempt to simulate as nearly as possible *in vivo* conditions, Schultz-Haudt and Scherp(3) used as substrate fresh bovine Achilles' tendon stored at –20°C and sterilized before use with ethylene oxide vapor for 18 hours at room temperature. Such treated tissue was deemed unaltered, since it appeared normal histologically when stained with hematoxylin and eosin, and resisted digestion by crystalline trypsin. The authors concluded that oral bacterial collagenases may indeed play a significant role in the breakdown of periodontal collagen. A similar technic has been used by Roth and Myers(4). Except for the occasional isolation of a *Clostridium* species, organisms able to lyse unaltered collagen were not found in cultures of the contents of either normal gingival sulci or periodontal pockets. The present experiments were undertaken to extend the previous work(3) to human gingival tissue.

Methods. Fresh human gingival tissue, obtained, without pretreatment, by gingivectomy on patients suffering from periodontitis simplex, was placed immediately in 10 ml of trypticase-casein-yeast-extract broth and incubated aerobically for 10 days, after which time the tissue and bacterial growth were removed by centrifugation and the supernatant fluid was analyzed for hydroxyproline, as a measure of collagenolysis(3). Representative samples of gingival tissue in each test varied in dry weight from 5 to 20 mg and contained from 30 to more than 160 μg of hydroxyproline. The minimum significant increase of hydroxyproline was considered to be 2 μg per ml of supernate, when compared to control broth cultures of the bacteria indigenous to the original tissue. Anaerobic incubation of the tests did not materially affect the results and was, therefore, employed only in the first experiments. In tests of samples from 21 patients, no collagenolysis could be detected (Table I). This resistance of the gingival collagen to digestion was confirmed by histological examination. After the supernate had been removed for hydroxyproline analysis, each piece of gingival tissue was fixed in formol-saline and embedded in paraffin. Sections were stained respectively with hematoxylin and eosin and by the Mallory-azan technic (Heidenhain's modification). For control, a sample of each specimen had been incubated for 10 days in sterile conditions, maintained by 200 i.u. of penicillin and 200 μg of streptomycin per ml. Fig. 1A shows that only slight cellular autolysis occurred in the control specimen, as seen in the epithelium and inflammatory infiltration of the underlying connective tissue. In contrast, Fig. 1B illustrates the complete disorganization of all tissue components, other than collagen and reticulin, produced by incubation with the gingival bacteria. The connective tissue in these specimens stained normally with the Mallory-azan method and was microscopically unaltered, although no fibroblasts were observed. As a control in each experiment, a sample of bovine Achilles' tendon, which had been stored in the frozen state and sterilized by ethylene oxide, was incubated in broth with the biota from the respective sample of gingival tissue. Collagenolysis was demonstrable in 15 of the 21 tests. Furthermore, the collagen of gingival tissue and tendon, sterilized with antibiotics, was shown to be digested by filtrates of broth cultures of *Clostridium histolyticum, i.e.*, true collagenase

dermis, the cellular infiltration of which is clearly visible. There is slight "loosening" of the stratum spinosum cells immediately adjacent to the stratum germinativum. (B) Section of human gingiva incubated for 10 days in broth with its indigenous flora. Complete disintegration of all elements other than the dermal connective tissue is apparent. (C) Bovine Achilles' tendon stored at –20°C and treated with ethylene oxide vapor for 18 hr. The fibroblasts are not well differentiated and the collagenous fibers appear homogeneous and swollen. (D) Bovine Achilles' tendon stored at –20°C. The fibroblasts are well differentiated and the collagen bundles appear compact. (E) Human gingival tissue treated with ethylene-oxide vapor for 18 hr. Homogenization is obvious. The dermal connective tissue appears "hyalinized" and merges with the stratum germinativum. Rete pegs can still be seen in the stratum spinosum. (F) Untreated human gingival tissue. All dermal and epidermal elements are clearly differentiated. (A) and (B), ×45, Mallory-azan stain; (C) and (D), ×75, Mallory-azan stain; (E) and (F), ×45, stained with hematoxylin and eosin.

TABLE I. Collagenolytic Activity of Human Gingival Bacteria on Human Gingival Tissue and Bovine Achilles' Tendon.

Series	Tissue	Treatment	Organisms*	No. positive / No. tests	Control tendon†
1	Gingiva	None	Indigenous (10 days)	0/21	15/21
	"	Antibiotic sterilization	*Cl. histolyticum* filtrate (3 days)	6/ 7	
	Tendon	*Idem*	*Idem*	1/ 1	
	Gingiva	None	Indigenous (6 wk)	3/ 5	5/ 5
2	Tendon	Freezing and ethylene oxide	Subgingival scrapings‡ (10 days)	8/11 (J.T.) 13/19 (S.–H.)	
3	Gingiva	None	Indigenous (10 days)	0/ 8	7/ 8
	"	Freezing and ethylene oxide	" "	5/ 8	

* Aerobic incubation.

† Stored in the frozen state, sterilized by ethylene oxide, and incubated with microbiota from respective untreated gingival tissue.

‡ From cases of chronic marginal gingivitis. J.T. denotes results of present experiments. S.–H. denotes previously reported results(3).

(Table I). Continued incubation of 5 randomly selected tests for 6 weeks increased the breakdown of tendon in all cases and liberated a small but significant amount of hydroxyproline-containing material in 3 of the 5 previously negative tests of untreated gingival tissue. Finally, we confirmed the results of Schultz-Haudt and Scherp(3) by showing that broth cultures of the crevicular microbiota from 8 of 11 subjects with localized chronic marginal gingivitis lysed the collagen of tendon that had been frozen, thawed, and sterilized by ethylene oxide (Table I). These results indicated that such treatment rendered collagen susceptible to bacterial proteases other than collagenase.

Results. To test this conclusion, specimens of gingival tissue were divided into halves, one of which was incubated immediately in a tube of broth with its indigenous microbiota. The other was placed in a sterile tube, held at –20°C for 72 hours, thawed, and sterilized by exposure to ethylene oxide vapor for 18 hours; broth was added aseptically and inoculated with the growth from the first tube. The sterilized halves underwent collagenolysis in 5 of 8 tests, compared with 7 of 8 tendon controls, whereas none of the untreated halves did so (Table I). The alteration of the substrate in these experiments could be due to denaturation by freezing and thawing, chemical action of ethylene oxide, or a combination of these factors. These possibilities were explored in 15 additional experiments, in which the specimens of gingival tissue were divided into quarters, which were transferred to sterile tubes and treated as follows: (1) incubated immediately with its indigenous flora in 10 ml of broth added aseptically; (2) held at –20°C for 3 days; (3) sterilized by immediate exposure to ethylene oxide vapor for 12 hours; and (4) held at –20°C for 3 days, thawed, then sterilized by ethylene oxide vapor at room temperature for 12 hours. In 4 cases, sufficient tissue was available to provide a fifth portion, which was sterilized by immediate submersion in acetone at –8°C, transfer after 12 hours to a sterile tube, and evaporation of the residual acetone at 37°C for 12 hours(1). Each test included as a control a tube containing a sample of bovine Achilles' tendon, previously frozen, thawed, and sterilized by ethylene oxide. At the conclusion of the respective treatments to tubes 2 *et seq.*, broth was added aseptically, inoculated with the growth from tube 1 and incubated aerobically for 10 days.

Once again, collagenolysis of the untreated gingival tissue was not demonstrable (Table II). Freezing or treatment with ethylene oxide rendered the collagen slightly susceptible to digestion in a few cases, but the combination of these procedures was far more effective. Gingival collagen sterilized by ace-

TABLE II. Effect of Freezing or/and Ethylene Oxide on Susceptibility of Gingival Tissue to Collagenolysis by Indigenous Gingival Bacteria.

Tissue	Treatment	No. positive / No. tests*
Gingiva	None	0/15
"	Freezing	2/15
"	Ethylene oxide	4/15
"	Freezing & ethylene oxide	12/15
Bovine tendon	*Idem*	15/15
Gingiva	Acetone	0/ 4

* All cultures incubated aerobically for 10 days.

tone, however, resisted digestion. In this series, all of the control tests of tendon resulted in collagenolysis.

Alterations of collagen by freezing and ethylene oxide were demonstrated also by histological means. Fresh specimens of human gingival tissue and bovine Achilles' tendon were stored at −20°C for 3 days, thawed, sterilized with ethylene oxide vapor as described previously, fixed in formol-saline, and embedded in paraffin. Sections were stained with hematoxylin and eosin and by the Mallory-azan technic. The treated tendon stained with hematoxylin and eosin revealed no difference in staining properties, although under high power the collagen fibers appeared swollen and homogeneous with either stain (Fig. 1C), when compared to those of untreated tendon (Fig. 1D). Similarly treated and stained human gingival tissue, however, showed complete homogeneity of the connective tissue elements and loss of differentiation between the basal cell layer and the underlying connective tissue (Fig. 1E and F). The Mallory-azan technic revealed striking differences between the treated and untreated tissues. In both treated tendon and gingival tissue there was a loss of the blue stain characteristic of normal collagen. Instead, the orange G was largely retained throughout the dense collagenous elements of the tissues. Prolonged exposure to the anilin blue-orange G reagent (1½ hours or longer) in most cases reversed the staining of the connective tissue toward normal, although even after this time a slight background of orange G was still apparent. Also, the epithelium of the treated gingival tissue took the orange G much more intensely. On the other hand, cellular elements such as erythrocytes and inflammatory cells stained normally and so did the loose reticular fibers that surround the blood vessels, etc., and form the connective tissue support of the basement membrane of the gingival tissue. The observed changes were probably attributable to the action of ethylene oxide, for they were identical in gingival tissue treated with ethylene oxide without prior freezing (Fig. 1E).

Discussion. In 3 series of tests on specimens of untreated gingival tissue from 44 patients with sufficiently severe periodontitis simplex to warrant gingivectomy, the gingival collagen resisted digestion during a 10-day period of incubation with those members of the indigenous gingival microbiota that grew under the conditions of our experiments. After 6 weeks' incubation, 3 of 5 additional tests showed only slight collagenolysis. The discrepancy between these results and those previously reported(3) can be explained by the fact that the collagen becomes susceptible to proteases of the gingival microbiota when frozen, thawed, and sterilized by ethylene oxide, as in the prior study. However, it still resists crystalline trypsin(3).

The extreme resistance of native collagen to enzymatic degradation has been thoroughly documented. Thus, Neuman and Tytell(5) found that only *Clostridium perfringens, Cl. histolyticum,* and pepsin attacked all of the various collagenous substrates they tested and emphasized that the degree of enzymatic degradation was proportional to the degree of denaturation that the collagen had undergone during its preparation. MacLennan *et al.* (6), using sterile rabbit Achilles' tendon as a native collagenous substrate, could find evidence of collagenase production by only *Cl. perfringens* and *Cl. histolyticum.* Except for the rare occurrence of *Clostridium* species, the gingival microbiota has not been shown to produce a true collagenase. Consequently, a role for this enzyme in periodontal disease has not been substantiated. However, it is almost certain that many species of this microbiota did not grow under the conditions used and the possibility remains that these organisms, alone or in combination, can effect collagenolysis. There is ample evidence of oral bac-

terial proteases, other than collagenase, capable of attacking altered collagen(1,2,4,7). The slight collagenolysis observed after prolonged incubation of untreated gingival tissue with its indigenous microbiota may also be secondary to substrate alteration. It seems reasonable to suggest, therefore, that the loss of collagen fibers characteristic of periodontal disease results not from the primary action of microbial collagenase but from an alteration of the collagen which renders it digestible by other proteases of the resident crevicular flora, in the manner postulated by Sherry *et al.*(8). However, no mechanism has been demonstrated whereby such an alteration could occur *in vivo*.

Ethylene oxide has been recommended for sterilization of sundry materials and culture media and it has been generally assumed to be relatively benign. Judge and Pelczar(9) found that it had no detrimental chemical effect on a series of carbohydrates. Recent work by Windmueller *et al.*(10), however, shows that ethylene oxide renders casein nutritionally ineffective for rats. According to Fraenkel-Conrat(11), 1,2 epoxides reacted readily with the carboxyl and basic groups of proteins in aqueous solution. However, their binding capacities for an acid dye (orange G) were undiminished (*cf.* also Einbinder and Schubert(12) on the binding of dyes by collagen under different ionic conditions). This fact, and the absence of a comprehensive theory of the tri-acid staining methods for connective tissue make it difficult to rationalize the altered staining reactions observed in tissue treated with ethylene oxide. In any event, the net effect was to decrease the rate of displacement of orange G from the collagen by the less strongly acid anilin blue. Our results appear analogous to the recent observations of Fullmer and Lillie(13) who report altered staining reactions of collagen with orcein following blockade of polar groups of the collagen by dinitrofluorobenzene, benzoylation, methylation, and acetylation.

Summary. In 44 tests, in which untreated human gingival tissue from cases of periodontitis simplex was incubated for 10 days in broth with its indigenous microbiota, no breakdown of collagen could be detected either chemically or histologically. All other tissue elements, however, were stripped away. In concurrent controls, bovine Achilles' tendon, stored frozen and sterilized by ethylene oxide as in a previous investigation, was used as a substrate for the same microorganisms and underwent collagenolysis in 37 of 44 tests. Freezing, thawing, and sterilization of human gingival tissue with ethylene oxide rendered its collagen susceptible to degradation by its indigenous flora in 17 of 23 tests. This action of ethylene oxide was paralleled by a striking alteration of the reaction of the tissues to the Mallory-azan stain for connective tissue. The participation of true bacterial collagenases in periodontal disease has not yet been substantiated. Conclusions to the contrary have been based on the digestion of altered collagen by other proteases.

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