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DEGRADATION OF DERMAL FIBRILLAR STRUCTURES: EFFECTS OF COLLAGENASE, ELASTASE, DITHIOERYTHRITOL AND CITRATE

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Abstract. Clostridial collagenase, elastase, medium material of human skin culture, dithioerythritol and citrate buffer, pH 3.5, were applied to fresh human skin specimens. Phenomena of degradation of dermal fibrils were observed by electron microscopy.

Collagen fibrils. Clostridial collagenase and culture medium material produced two types of collagen fibril degradation, viz. filament bundles with cross bands, and twisted and tapered collagen fibrils. Under the electron microscope, the final degradation product of both appeared as a lattice-like structure. The fibrils disintegrated into finer filaments under the influence of citrate buffer, while elastase and dithioerythritol did not affect collagen fibrils at all.

Elastic tissue. The matrix of elastic fibres was influenced by elastase, the fibrils by dithioerythritol. The latter were transformed into smooth-surfaced, dense, beaded strings.

Dermo-epidermal junction. Clostridial collagenase dissolved anchoring filaments, basal lamina and anchoring fibrils. The extracellular dense lamella of semidesmosomes appeared as a dotted line. Elastic fibrils remained unchanged. Elastase dissolved all junction structures except anchoring fibrils. The culture medium material transformed the basal lamina and the anchoring filaments into granular structures. The anchoring fibrils were unaffected, but were separated from the basal lamina by a lucent band. Elastic fibrils anchored to the basal lamina also remained unaffected. Dithioerythritol destroyed the basal lamina, the anchoring filaments and the extracellular lamella of semi-desmosomes. The anchoring fibrils were unchanged.

Key words: Degradation; Collagen fibrils; Elastic fibres; Dermal-epidermal junction

Hitherto, ultrastructural degradation of dermal connective tissue has been poorly explained. By light or electron microscopy, normal collagen fibrils are often seen to be phagocytized by macrophages (21). Peculiar filament bundles in the dermis are thought to represent degraded collagen fibrils (14). Dermo-epidermal separation is seen in certain skin diseases. Using some degrading agents, previous experimenters found tapered ends after clostridial collagenase (17), disappearance of elastic fibre matrix with elastase (2), complete removal of elastic fibrils by dithioerythritol and guanidine (2), and dissolution of the basal lamina by clostridial collagenase (16). However, the degradation of these dermal components has not been studied in ultrathin sections.

This study revealed ultrastructural changes of dermal fibrillar components produced by various degrading agents. It aimed at elucidating the nature of the dermo-epidermal junction.

MATERIAL

Fresh samples of human skin were obtained by plastic surgery on breasts. Using a 3 mm punch, skin specimens of split thickness were prepared under sterile conditions.

Crude clostridial collagenase (Worthington, Type I, 125 units/mg) was dissolved to a concentration of 2 mg/ml of physiological saline, pH 6.8, Hanks' balanced salt solution, pH 7.2, or Tris-HCl buffer, pH 7.6, with 0.005 M $CaCl_2$.

Purified clostridial collagenase (Worthington, Type IV, 375 units/mg) was dissolved to a concentration of 0.7 mg/ml of the same solvents as described above.

Elastase (from hog pancreas, Sigma) was diluted five times with 0.2 molar borate buffer, pH 8.0, with 0.01 molar calcium acetate.

Citrate buffer, 1 molar, pH 3.1.

Dithioerythritol (Sigma) was dissolved in Hanks' balanced salt solution, pH 7.4, to a concentration of 10 mg/ml.

Culture medium material was prepared as follows (10, 30). Small specimens of fresh human skin were incubated in Eagle's minimum essential medium with Earle's salt supplemented by penicillin (5000 units/ml), streptomycin (5000 μg/ml) and L-glutamine (200 mmol), 1 ml of each

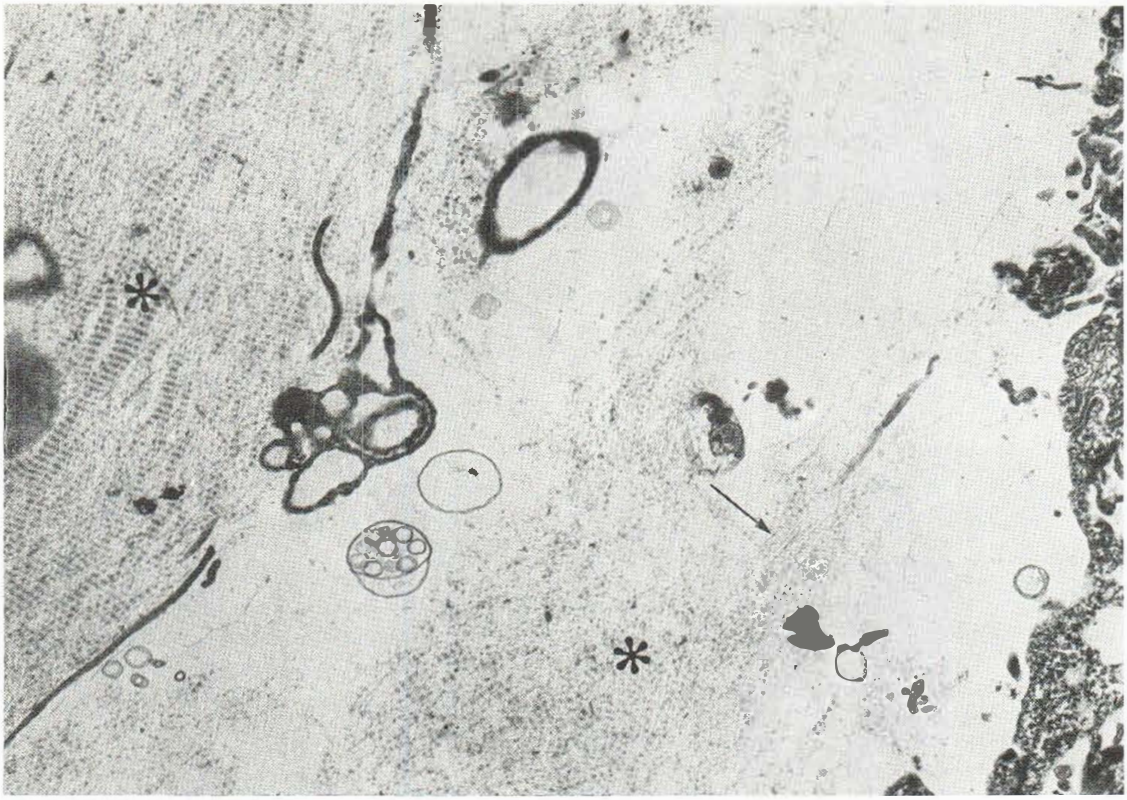


Fig. 1. Skin specimen affected by clostridial collagenase, pH 6.8, for 6 hours. Dermal collagen fibrils are replaced by filament bundles with cross-bands (*) and lattice. Elastic fibrils are unaffected (→). The dermo-epidermal junction has disappeared. ×8 000.

per 50 ml at 37°C. The incubation was continued for 5 days. The medium was changed daily and stored in a deep-freezer after mixing with an equal volume of Tris-HCl buffer, pH 7.4, with $CaCl_2$ 0.005 M. The medium was centrifuged at about 45 000 *g* for one hour. The supernatant was precipitated by ammonium sulphate. Samples saturated to varying degrees, between 30 and 50%, were collected and dissolved in small amounts of distilled water. The solution was dialysed against distilled water and freeze-dried. The powder was stored in a deep-freezer before use. Ten mg powder was dissolved in 1 ml of Hanks' balanced salt solution, pH 7.2 and Tris-HCl buffer, pH 7.6.

METHODS

Three pieces of skin were placed in each incubation medium and incubated at 37°C. The specimens to be incubated in the culture medium material were frozen overnight. The incubation media were filtered once through a Millipore filter (pore size 0.22 μm). The incubation periods were 3 or 6 hours for both crude and purified clostridial collagenase, 6 hours for elastase and dithioerythritol, and 12 and 24 hours for the culture medium material. For control, plain solvents and boiled solutions of the enzymes were used. As an inhibitor, EDTA was used in the collagenase solution at a concentration of 2 mg/ml.

After incubation, the specimens were fixed in a 6%

Fig. 2. Twisted dermal collagen fibrils with tapered ends after 6 hours' incubation in clostridial collagenase, pH 7.6. ×40 000.

Fig. 3. Fresh skin specimen after 3 hours' incubation in clostridial collagenase, pH 6.8. Filament bundles with cross-bands (→) and lattice patterns (*) are seen. Elastic fibrils and matrix (*E*) are unaffected. ×80 000.

Fig. 4. Fresh skin after 3 hours' incubation in clostridial collagenase, pH 6.8. Remnants of basal lamina are seen facing semi-desmosomes (→). Unaffected elastic fibrils (*E*) are continuous with the basal lamina. Elsewhere, lattice and filament bundles with cross-bands (*F*) replace collagen fibrils. ×40 000.

Fig. 5. Fresh skin after 3 hours' incubation in clostridial collagenase, pH 6.8. Remnants of basal lamina are indicated by asterisks. Arrows indicate comb-like semi-desmosomes. ×40 000.

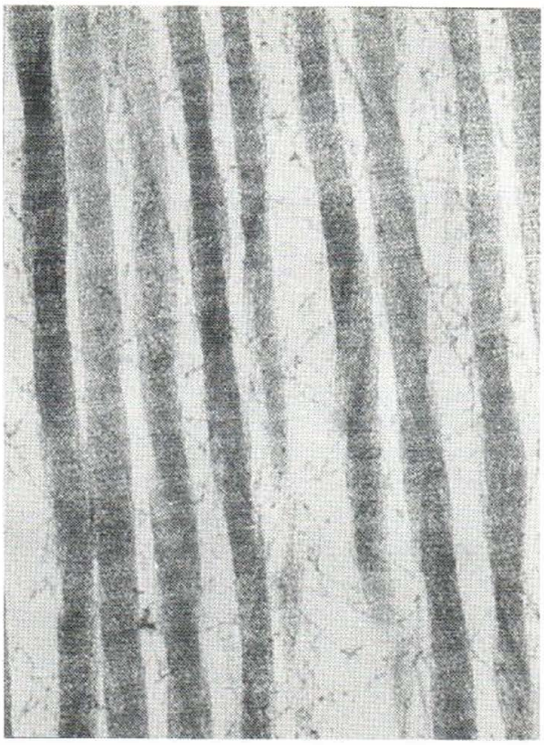
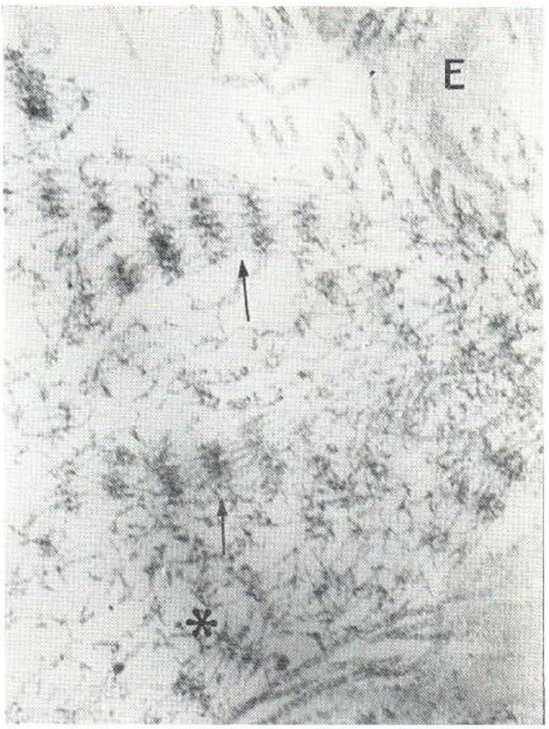


Fig. 2



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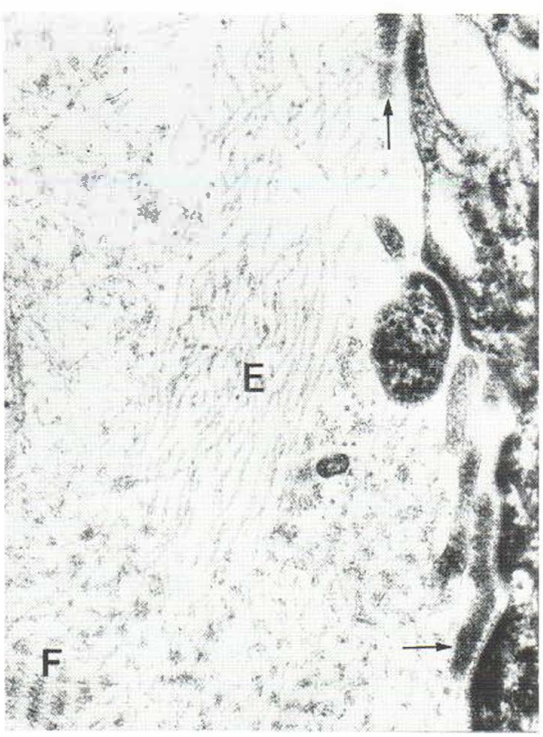

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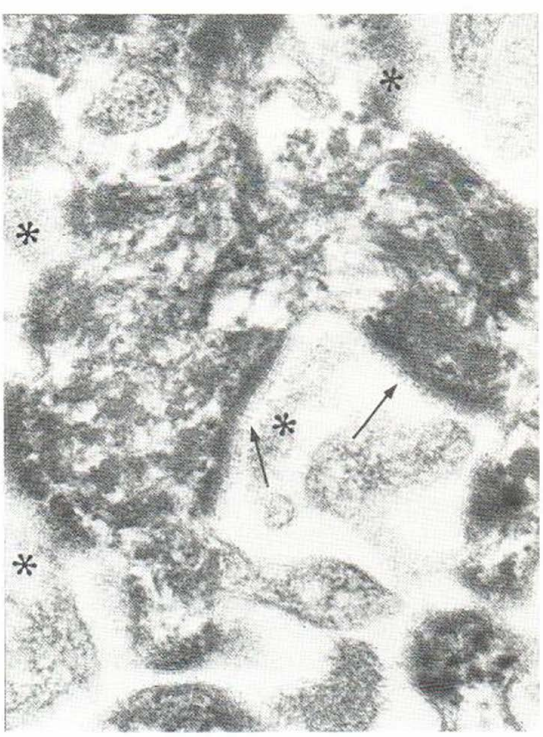

Fig. 5

Fig. 6. Effect of elastase for 6 hours at pH 8.0. Collagen fibrils (*C*) and elastic fibrils (→) are unaffected. Masses of elastic fibrils (*E*). Basal lamina has disappeared. ×20 000.

glutaraldehyde solution in cacodylate buffer, pH 7.4, with 7.5 % sucrose without washing. Thereafter, the specimens were osmicated, dehydrated and embedded in Epon 812. The ultrathin sections were stained by uranyl acetate/lead citrate, ruthenium red (20), and by the periodic acid silver proteinate technique (32). The sections were studied by a Siemens electron microscope (Elmiskop 1A) and a Jeol 6Y electron microscope at 80 kV.

OBSERVATIONS

Clostridial collagenase. The crude enzyme dissolved the dermo-epidermal junction and the collagen fibrils. Six hours of incubation transformed the specimens into a suspension of undissolved remnants—epidermal sheaths, elastic fibres, vessels and ducts (Fig. 1). After 3 hours of incubation, the dermal collagen fibrils were replaced by a lattice intermingled with twisted collagen fibrils (Fig. 2) or filament bundles with cross-bands (Fig. 3). The twisted fibrils showed a twisting angle of 15° to the fibril axis and normal axial periodicity. The ends of the fibrils were tapered. The cut-surfaces showed defective contours. The cross-banded filament bundles varied in length and width, showing repeating units consisting of 33–37 nm wide dense bands at 46–50 nm intervals. The intervals showed 2.5 nm thick filaments running parallel to the axis. The bands were stained by the periodic acid silver proteinate technique (32), while they remained unstained by ruthenium red. The lattice formed fine meshes in between these abnormal fibrillar structures. The filament bundles appeared in the enzyme solutions of pH 6.8–7.2, and the twisted fibrils in the pH 7.6 solution. The parts of the basal lamina which were opposed to the semi-desmosomes appeared to be more resistant to the influence than the rest of the lamina (Fig. 4). The extracellular lamella of the semi-desmosomes (*H'*-line; 29) appeared as a dotted line, while the cell membrane and the attachment plaque were preserved (Fig. 5). The elastic fibrils were left free in the junction (Fig. 4).

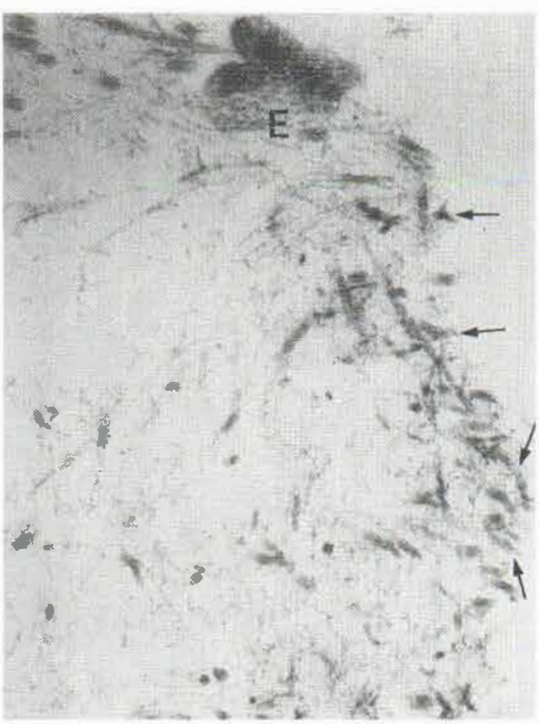

Fig. 7. Dermo-epidermal junction of the same specimen as shown in Fig. 6. Basal lamina has disappeared. Elastic fibrils (*E*) and anchoring fibrils (→) remain unaffected. ×40 000.

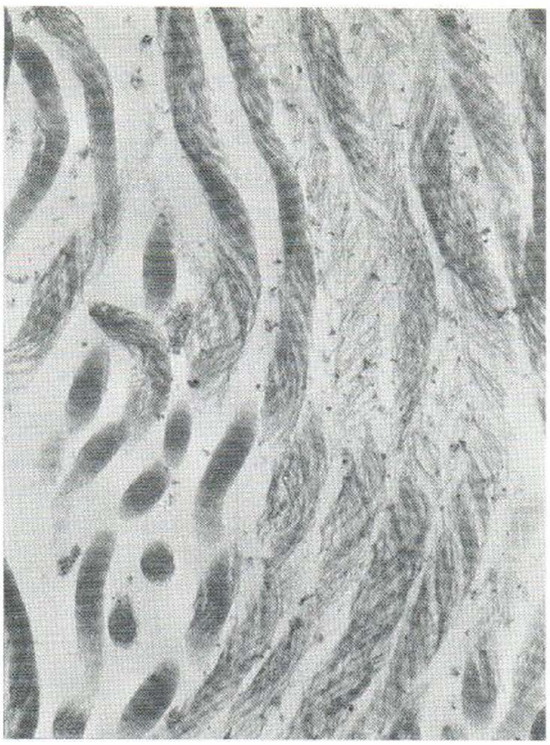

Fig. 8. Dermal collagen fibrils incubated in citrate buffer, pH 3.1, for 24 hours. Collagen fibrils show distinct twisting, while no lattice pattern is seen between the fibrils. ×40000.

Purified clostridial collagenase produced identical degradation of the dermal fibrils and the dermo-epidermal junction. Boiling of the enzyme solution or EDTA supplement prevented the forming of these abnormal fibrillar structures.

Elastase. Elastase dissolved the elastic fibre matrix, the basal lamina, the anchoring filaments and the so-called *H'*-line of semi-desmosomes (Figs. 6, 7). The anchoring fibrils and the elastic fibrils resisted this action, though the cross-bands of the anchoring fibrils often appeared blurred. The collagen fibrils remained unchanged. A boiled solution of the enzyme exerted no effect on these structures.

Citrate buffer. This buffer produced twisting of the collagen fibrils, the twisting angle being 30° to the axis (Fig. 8). The filaments of the fibrils appeared to be 8.3 nm in diameter and greater than 160 nm in length. The axial periodicity became indistinct when the fibrils were split longitudinally. No lattice was seen in the interfibrillar spaces. Elastic fibrils appeared straightened, though the matrix was unchanged.

Dithioerythritol. Dithioerythritol destroyed the basal lamina, anchoring filaments and elastic fibrils, as well as the extracellular part of the semi-desmosomes (Fig. 9). The attachment plaques and cell membranes remained unaffected. The basal lamina lost its normal filamentous structure and appeared as amorphous pieces. The anchoring filaments were seen ruptured and retracted, forming structure-less masses on the basal cell membrane, together with destroyed semi-desmosomes (Fig. 9). The elastic fibrils appeared as smooth, dense, beaded strings (Fig. 10). The collagen fibrils, the anchoring fibrils, and the elastic fibre matrix remained unaffected.

Culture medium material. The material produced either filament bundles or twisted fibrils, as seen

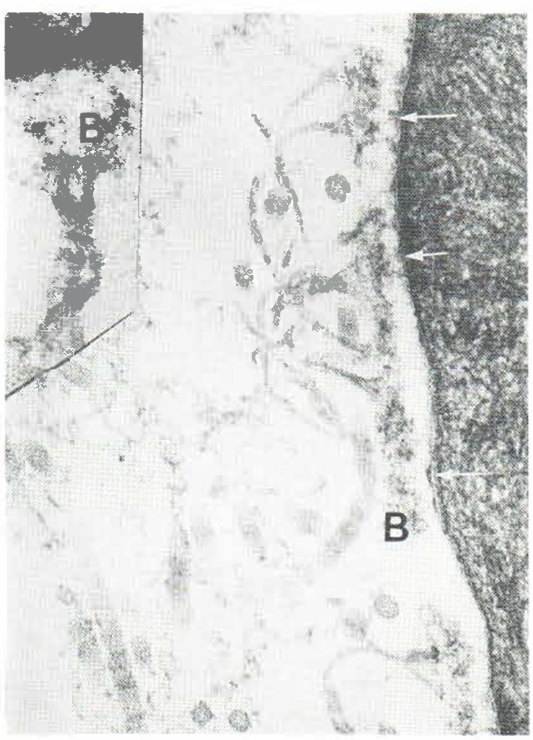

Fig. 9. Dermo-epidermal junction after 6 hours' action of dithioerythritol. Anchoring fibrils are intact, while the basal lamina (*B*) is transformed into dense, amorphous pieces. Anchoring filaments are broken and have formed dense masses on the basal cell membrane (→). ×40000. Inset indicates an anchoring fibril with distinct bands, while the basal lamina (*B*) has almost disappeared. ×80000.

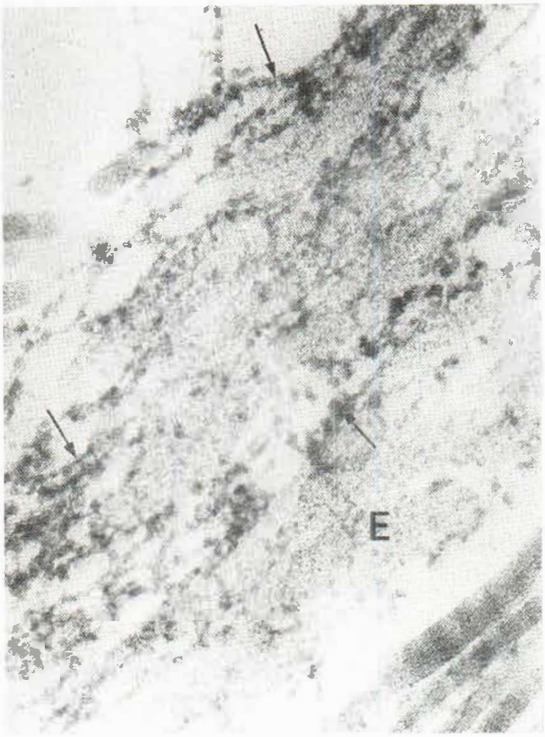

Fig. 10. Dermal elastic fibre after 6 hours' action of dithioerythritol. The matrix (*E*) is not attacked but the fibrils are transformed into dense, beaded strings (→). ×40 000.

after clostridial collagenase treatment (Figs. 11, 12). The elastic fibre matrix and fibrils were not attacked. In the dermo-epidermal junction, the anchoring filaments disappeared (Fig. 13). The *H'*-lines were destroyed. The basal lamina appeared granular, with a thickness of about 85 nm, still keeping its continuity. The anchoring fibrils were separated from the lamina by a lucent zone, but they retained their distinct cross-bands (Fig. 13). The elastic fibrils preserved their continuity with the basal lamina. A boiled solution exerted no effect on the dermis.

DISCUSSION

Clostridial collagenase is a protease which digests native collagen fibrils, gelatin and an acid extract of collagen fibrils at physiological pH and temperature, but no other type of denatured collagen (26). Native collagen fibrils are resistant to proteolytic enzymes other than collagenase. However, the fibrils are easily denatured by physical and chemical agents, for instance heat and grinding. When denatured, the proteases may attack the fibrils (12, 26). To preserve collagen fibrils in their native condition as embedded in the other tissue components, the present authors chose to use punched specimens of fresh skin as substrate, despite the fact that the action does not take place evenly as it does on homogenates.

Using homogenates of rat tail tendon, Keech (17) demonstrated tapered ends of the native collagen fibrils in shadowed samples subjected to clostridial collagenase incubation at pH 7.6 for 18 hours. This phenomenon seems to be evidence of degradation. The present study demonstrated that collagenase destroys the native collagen fibrils from the surface, transforming them into a lattice, with twisted and thinner collagen fibrils as intermediate stages.

By applying a neutral solution of clostridial collagenase to the dermis of living animals for 30 min, Kahl et al. (16) showed an absence of degradation of collagen fibrils. This was probably due to a too brief incubation or an inhibitory action of living tissues.

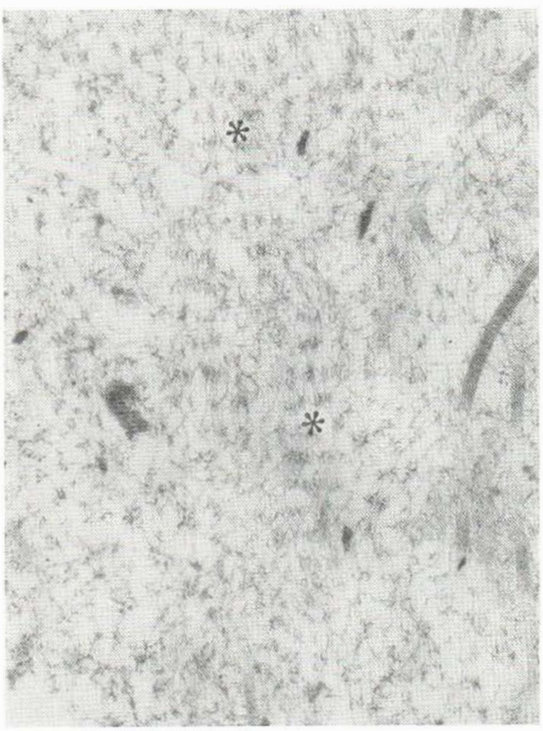

Fig. 11. Frozen skin specimen affected by culture medium material for 24 hours. Note lattice pattern and filament bundles with cross-bands (*). ×40 000.

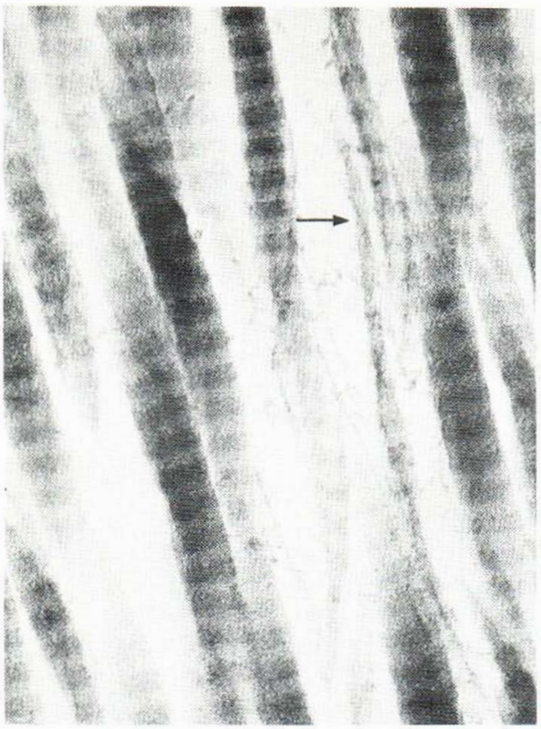

Fig. 12. Collagen fibrils after the action of culture medium material with Tris-HCl buffer, pH 7.6, for 24 hours. Twisting and tapered ends (→) are shown. ×40 000.

Fresh serum is known to contain collagenase inhibitor (26). The variations in the degradation may be due to the varying pH values of the enzyme solutions.

Among cellular proteases, under physiological conditions, collagenase is the only enzyme which is capable of degrading native collagen fibrils (1, 12, 26). The enzyme can be demonstrated in the medium of an epidermal cell culture (10, 14, 30). In whole-skin cultures, the dermis contains plenty of filament bundles with cross-bands replacing the collagen fibrils (14). The present study showed that this conspicuous structure represents collagen fibrils degraded by cellular collagenase. Acid mucopolysaccharides have been demonstrated in the cross-bands by fixatives containing ruthenium red (12). In the present investigation neutral mucoprotein is demonstrated in the bands, but no acid mucopolysaccharides. This disagreement may be due to unspecific contrasting of the ultrathin sections by lead compounds.

Filament bundles with cross-bands have been found in pathological dermis, e.g. in generalized scleroderma (21), localized scleroderma (22), scleromyxedema (9), amyloidosis (13), cancer (13), melanoma (13), and mycosis fungoides (23). The same phenomenon has been seen in the nucleus pulposus of prolapsed discs (31) and in discs of scoliosis (6). These findings suggest that cellular collagenase may take part in the disease processes. Perineural collagen fibrils of normal nerves and malignant nerve tumours have a structure which resembles these cross-banded filament bundles, although the structure details are not identical. Fibrils of normal perineurium are built up of more distinct cross-banded filaments and form spindles between collagen fibrils (27). In brain tumours, they contain an extra thin cross-line between the repeating bands (7, 25, 27).

Citrate buffer extracts of collagen fibrils were used as substrate in collagenase studies in vitro (26). This investigation indicated that the buffer can split native collagen fibrils into long filaments which are still susceptible to collagenase.

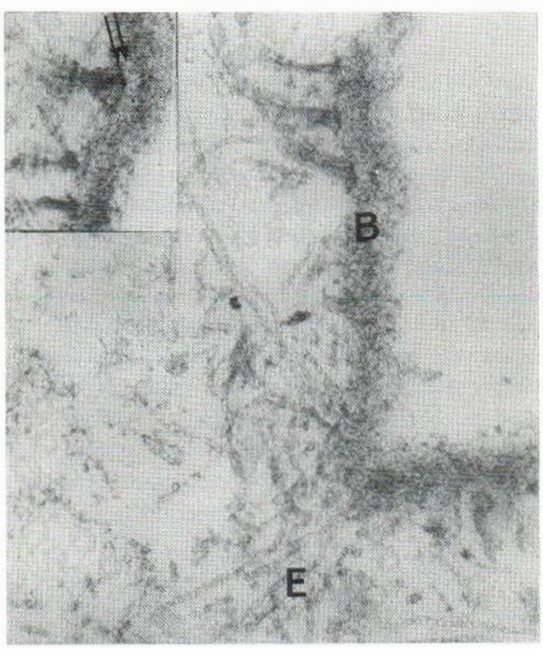

Fig. 13. Dermo-epidermal junction affected by culture medium material with Tris-HCl buffer, pH 7.6, for 24 hours. Thick, granular, basal lamina (*B*) maintains its continuity. Unaffected elastic fibrils (*E*) in continuity with the lamina. The anchoring filaments have disappeared. Inset shows three anchoring fibrils with distinct cross-bands, while a dense and a lucent band (→) separate the single fibrils from the basal lamina. ×40 000. Inset ×80 000.

Elastic fibres are composed of two morphological subunits, viz. matrix and fibrils (11, 19). The matrix is thought to precipitate in bundles of fibrils (19). In aged skin, the fibres usually lack the fibrils around the matrix (8). Elastic fibrils and matrix are more resistant to various denaturing agents than are collagen fibrils (26). Denatured elastic fibres are attacked by various proteolytic enzymes (26). Using a ligamentum nuchae homogenate pre-treated with 5 M guanidine and clostridial collagenase, previous investigators (2, 28) removed elastic fibrils completely from elastic fibres by means of dithioerythritol dissolved in 5 M guanidine. Dithioerythritol and its chemical conversion, dithiothreitol, reduces disulfide to sulfhydryl groups (4). The present study showed that dithioerythritol alone destroys the normal morphology of the elastic fibrils. Disulfide bonds are probably essential to the structure of elastic fibrils. Previous histochemical studies showed elastofibrils (5) and oxytalan fibres (3) in the dermis. These fibrils or fibres contained bonds which could be converted to SO_4-groups by oxidation. The fibrils had an ultrastructure which was similar to elastic fibrils (3, 21). The present study supplied evidence that these fibrils or fibres, though bearing a variety of names, are, in fact, identical.

Semi-desmosomes have a dense extracellular lamella to which anchoring filaments are bound (19). The lamella corresponds to the middle-line (M-line) of a desmosome (24) or to the attachment plaque (*H'*-line; 29). The M-line of a desmosome is influenced by dithioerythritol (15), whereas the line is resistant to trypsin (15) and the proteases used in this study. It would seem, therefore, that the extracellular lamella of semi-desmosomes is composed of anchoring filament material.

Ultrastructurally, the components of the dermo-epidermal junction, viz. basal lamina, anchoring filaments, anchoring fibrils and elastic fibrils, are interconnected yet lack specific connective structures (19). The chemical nature of these components is unknown. Presumably, the basal lamina is similar to the corneal and glomerular basal laminas which are known to contain collagenous protein, mucoprotein, as well as disulphide and hydrogen bonds (18). Its susceptibility pattern, as demonstrated in this study, suggests that the nature of the basal lamina and the anchoring filaments is similar to that of elastic fibrils and matrix, whereas the anchoring fibrils have no elastic characteristics. The differing susceptibility of the junctional components to clostridial collagenase and culture medium material (cellular collagenase) suggests that the collagenous protein in the dermo-epidermal junction is not identical with dermal collagen fibrils.

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