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ULTRASTRUCTURE OF FREEZE-CLEAVED DERMAL COLLAGEN

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Abstract. The collagen fibrils in human skin were studied by the freeze-fracture technique. The dermal collagen formed islands; individual fibrils were arranged in parallel, layered, or weaving patterns. The surface of individual fibrils was covered with amorphous or granular materials. In fixed specimens, a regular banding pattern was observed, repeating at intervals of 610–630 Å. One period consisted of an elevated band and a depressed band, each of 305–315 Å. When matched with native fibrils which were negatively or positively stained with phosphotungstic acid and/or uranyl acetate, the depressed band seemed to correspond to the c_1–d region and the elevated band to the a_1–b_2 region. Some fibrils demonstrated a twisting of the component protofilaments.

Previous ultrastructural studies concerning collagen have been made on thin sections of fixed tissues (4, 12), negatively or positively stained unfixed materials (10, 11, 13), purified and reconstituted materials (8, 10) or shadow-cast preparations (3, 13, 14). Most of these methods were designed to elucidate the internal macromolecular structures of individual fibrils but has severe limitations in the study of three-dimensional surface ultrastructure and the interrelationship between fibrils. The most commonly used material in these studies was animal tendon collagen and not human dermal collagen, except for that used in the shadow-casting studies of Tunbridge et al. (14), Grassmann et al. (3) and Hodge (10). Freeze-fracture or freeze-etch techniques allow one to replicate a large number of dermal collagen fibrils in their native state and in stereographic interrelationship.

In this report, the surface ultrastructure of the dermal collagen as revealed by these new techniques will be compared with the fine banded structure of native dermal collagen as revealed by phosphotungstic acid and/or uranyl acetate stains.

MATERIALS AND METHODS

Skin specimens of the palm and face were obtained from several normal individuals by biopsy under local anesthesia with 1% procaine. The specimens were immediately cut into 1 mm^3 cubes and fixed in 2% glutaraldehyde in 1/10 M cacodylate buffer, pH 7.4, for 3–5 hours and then rinsed in the same buffer overnight. Unfixed specimens were also used. No cryoprotective agents were used. A Denton DFE-2 freeze-etching apparatus with apposed specimen holders was used. Specimens were fractured at −170°C and either immediately cast with a mixture of platinum and carbon and then carbon alone, or etched by sublimation at −120°C for 1 min prior to replication. The details of this technique has been described in my previous publications (6, 7).

For a comparative study, parts of some specimens were teased in distilled water. Separated fibers were washed twice in distilled water, scooped up on Formvar-coated 200-mesh copper grids and stained with 1% phosphotungstic acid (pH 3.7) in distilled water for 15 min and then in 1% uranyl acetate in distilled water from 30 min to overnight. Some of the teased collagen on grids was lightly shadowed with platinum and carbon and observed direct. All specimens were examined in an Hitachi HU-12 electron microscope at 125 kV.

RESULTS

Freeze-cleaved human dermis showed bundles of collagen fibrils packed together to form islands of various sizes (Fig. 1). Each island was separated by ground substance and cellular components (Fig. 1). Within the same island individual fibrils usually ran in a parallel fashion (Figs. 1, 2*a*, 2*b*). In some islands, weaving or lattice-like patterns were observed (Fig. 3*b*). Collagen fibrils were coated with irregular, amorphous and often granular substances, particularly in unfixed specimens (Figs. 2*a*, 2*b*, 3*a*, 3*b*, 3*c*).

Both positive (Figs. 2*a*, 2*b*) and negative (Fig. 1) replicas of individual fibrils showed a distinct

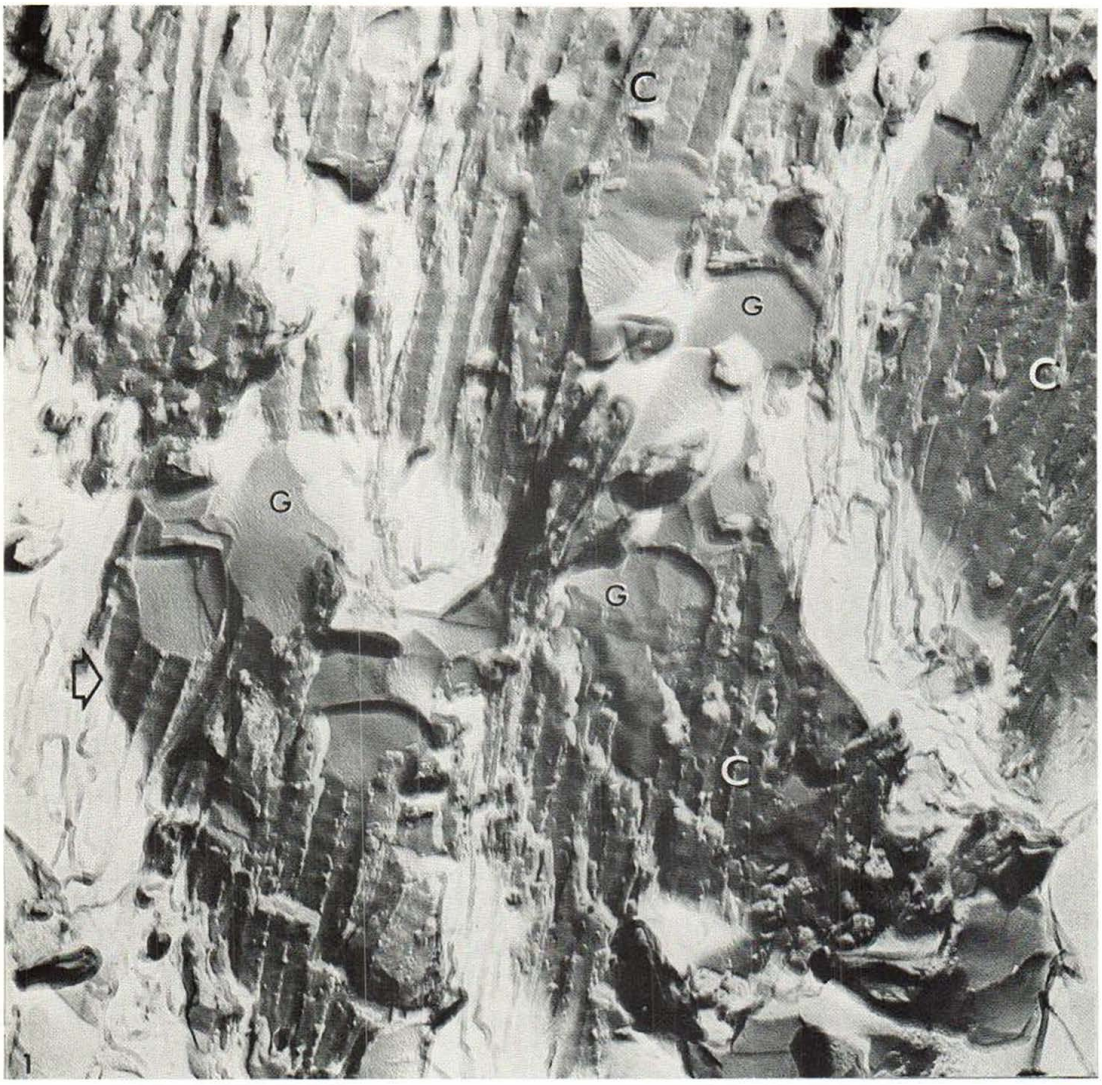


Fig. 1. Bundles of collagen fibrils (*C*) form islands. Individual islands are separated by ground substance (*G*). Positive as well as negative (*outline arrow*) imprints of collagen fibrils show a periodic banding pattern. Fixed facial skin, ×42 500.

periodic banding pattern which repeated at intervals of 610–630 Å. This pattern was clearly visible in fixed fibrils at a high magnification (Figs. 4*a*, 5*a*, 5*c*) but was often obscured in unfixed fibrils by a thick, coating substance. Each period consisted of a depressed interval or band of 305–315 Å and an elevated region of 305–315 Å (Figs. 5*a*, 5*c*). Extreme fluctuation of dimensions obviously caused by a very low shadowing angle was excluded from the measurements. In most instances, the elevated band and depressed band dimensions were approximately equal (Figs. 5*a*, 5*c*).

The teased collagen fibrils stained with phosphotungstic acid and uranyl acetate showed two different staining characteristics. The "negative" staining pattern consisted of a generally dense band (290–300 Å), i.e., hole zone (10), and a generally light band (290–300 Å), i.e., overlap zone (10). Within both zones there were a few asymmetric (or polarized) striations (Figs. 2*c*, 4*b*, 5*b*, 5*c*, 5*d*). The positive staining pattern showed most of the conventionally designated bandings which were also asymmetric (Fig. 5*f*). In both types of staining, the major banding pattern was repeated at intervals of 580–600 Å. The variation of positive and negative staining seemed to be caused by the degree of uranyl

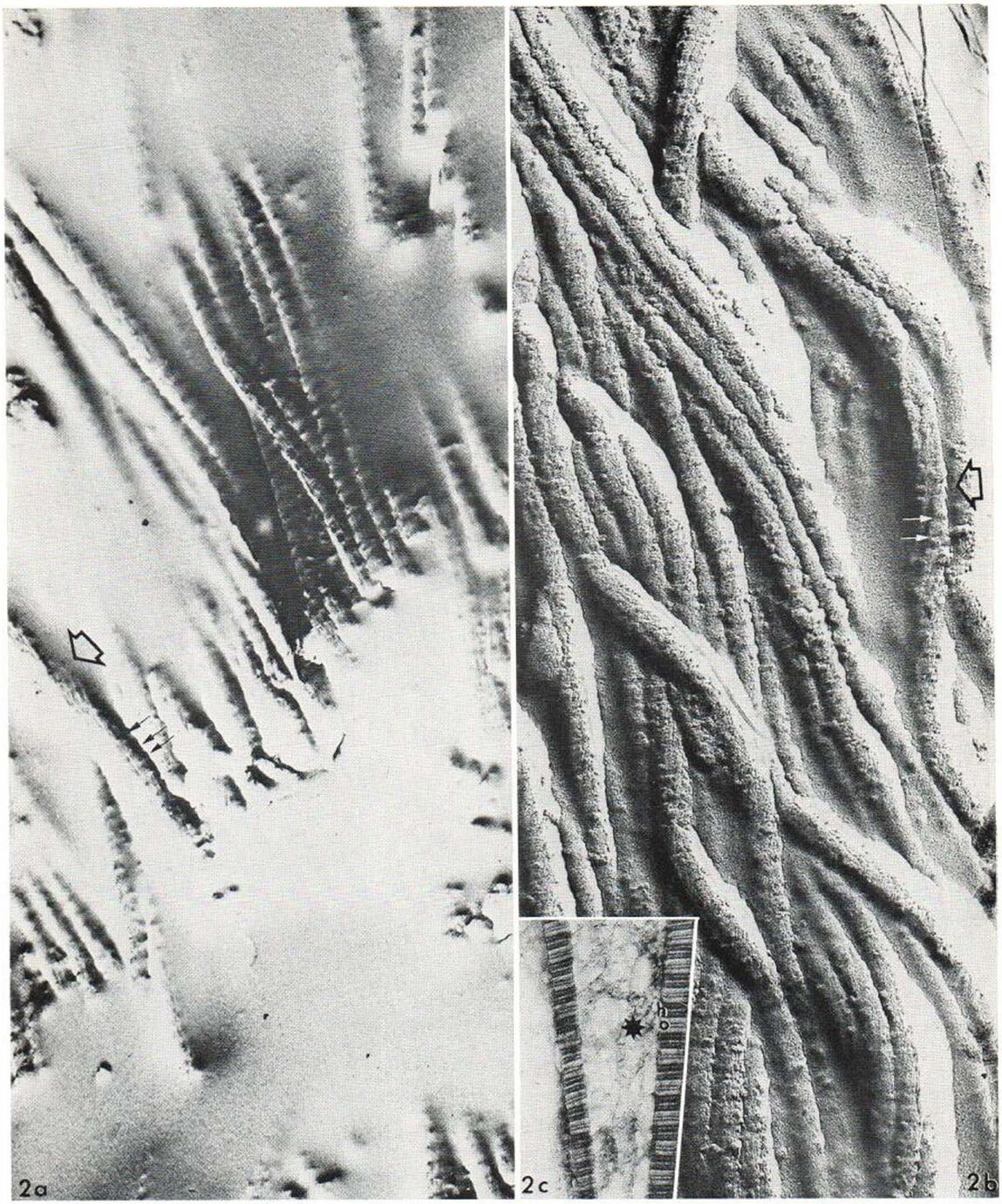


Fig. 2. (*a*) Collagen fibrils are bundled in parallel alignment. Not only a periodic banding pattern (*thin arrows*) but also twisting of the component filaments (*outline arrows*) are seen in the same fibril. Unfixed skin of the palm, × 42 900. (*b*) Structureless or fine granular substances coat the collagen fibrils. Twisting of the component filaments can be seen (*outline arrows*) in some fibrils which also show periodic striations (*thin arrows*). Unfixed skin of the palm, × 60 600. (*c*) Negatively stained native collagen fibrils are covered with an amorphous coat (*), which may correspond to the structureless substances coating the freeze-fractured collagen (Fig. 2*b*). Each fibril shows alternating dense hole zones (*h*) and light overlap zones (*o*) of approximately equal intervals. It is seen that the surface periodicity of the freeze-fractured collagen fibril roughly corresponds to that of these dense and light bands (see Figs. 5*a*–5*f* for further details). Skin of the palm, × 60 600.

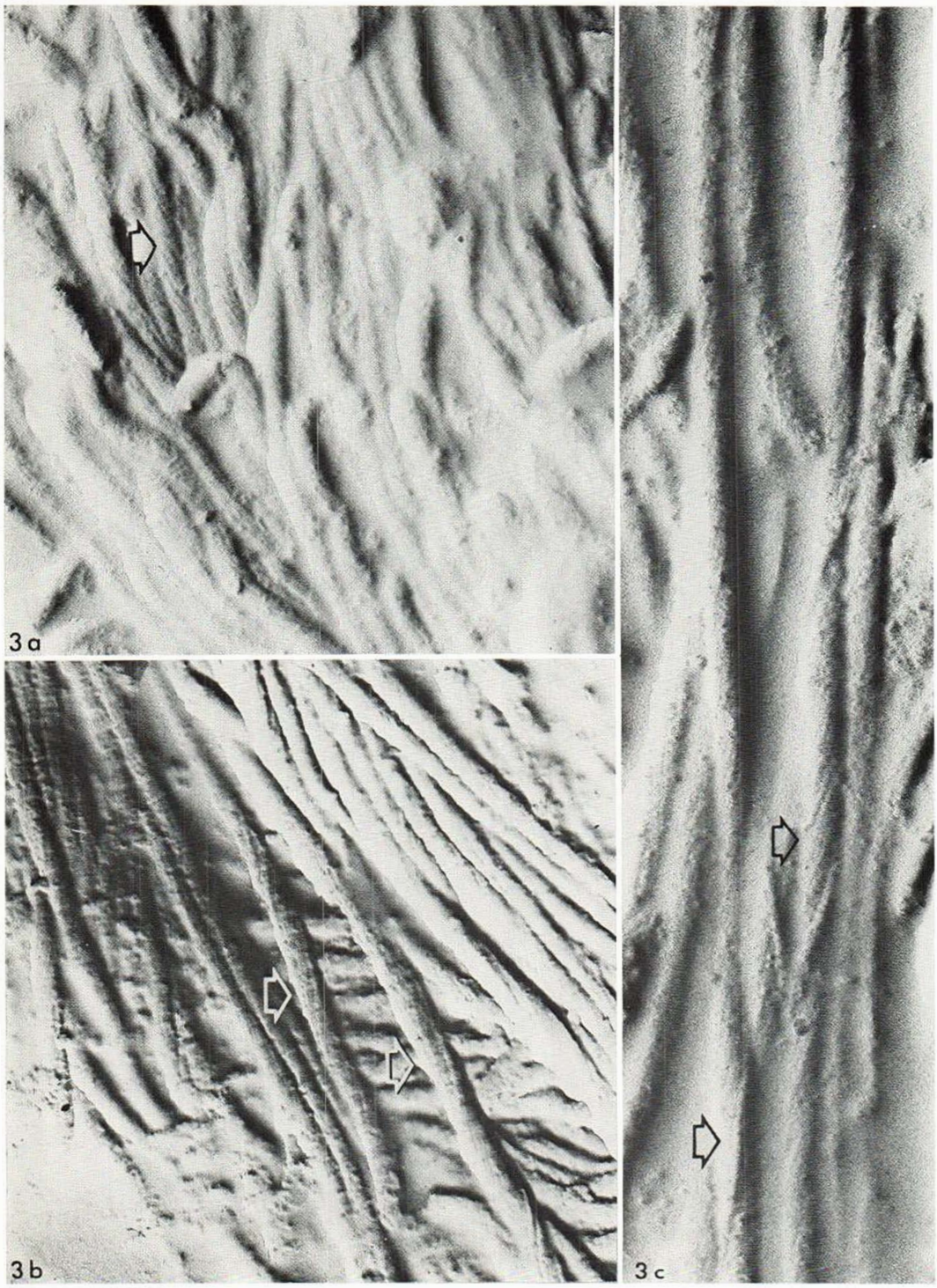


Fig. 3. (*a–c*) Irregularly bent or crooked fibrils in 3*a* are heavily covered with amorphous ground substances. Laminated or woven pattern of fibrils is seen in 3*b*. Twisting of component filaments (*outline arrows*) is obvious in many fibrils. Unfixed skin of the palm, 3*a* and 3*b*: × 44 250. 3*c*: × 59 750.

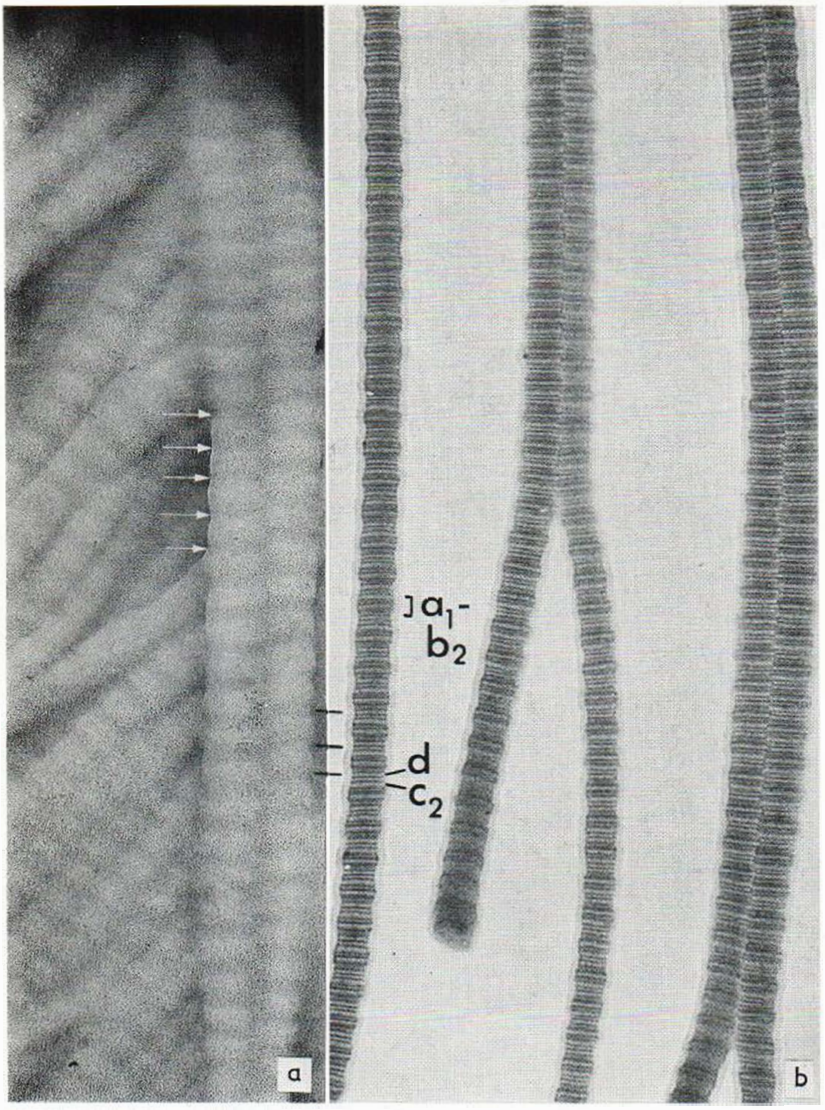
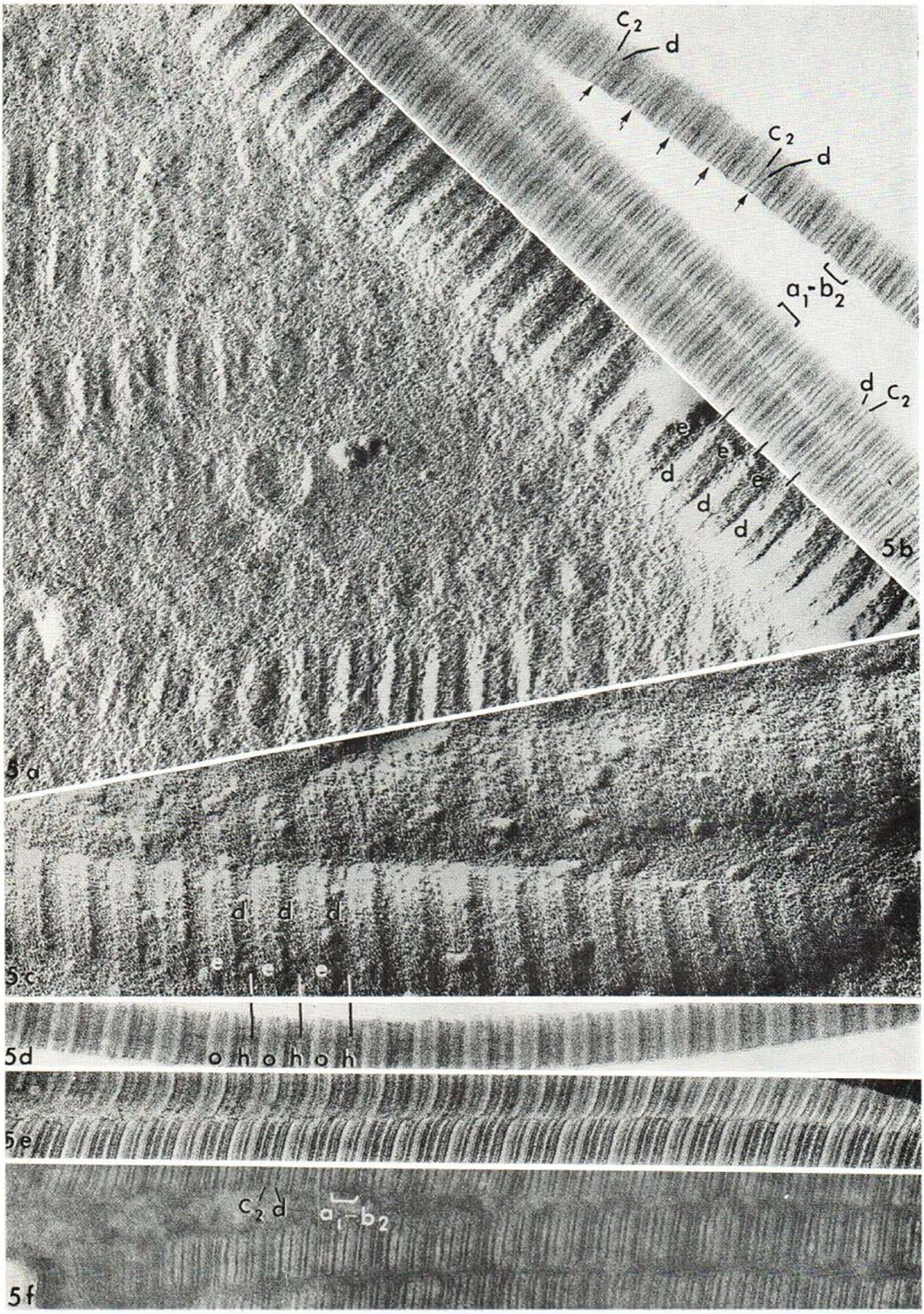


Fig. 4. (*a*) This replica definitely shows that an alternating elevation and depression (*arrows*) is the basis of the surface periodicity. Unfixed skin of the palm, × 75 000. (*b*) Lateral contour of negatively (partially positively) stained native collagen fibrils shows alternating surface elevation and depression. When matched with the replica fibril, the elevations and depressions seem to correspond very well. From the partially stained bandings it may be seen that the depressed zone corresponds to the c_2-d region and the elevated area to the a_1-b_2 region. Skin of the palm, × 75 000.

acetate penetration into individual fibrils. Transitional patterns between two stains were observed in some fibrils (Fig. 5*e*) and this helped in linking the negatively stained banding pattern to the individual bands of positively stained fibril.

When the lateral contour of both positively (Fig. 5*f*) and negatively (Figs. 4*b*, 5*b*) stained fibrils was followed, elevation and depression could often be detected. The depressed area approximately matched c_2-d region (Figs. 4*a*, 4*b*, 5*a*, 5*b*) and c_2-d region in turn corresponded to the dense band (hole zone) of the negatively stained fibrils (Figs. 5*f*→5*c*). The elevated area roughly corresponded to the a_1-b_2 region (Fig. 4*b*) or to the light band (overlap zone) of negatively stained fibrils (Figs. 5*f*→5*c*). The elevation (290–300 Å) and depression (290–300 Å) of negatively stained fibrils visually matched particularly well with similar elevation (305–315 Å) and depression (305–315 Å) of the freeze-etched fibrils when the latter picture was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photographically reduced slightly (Figs. 5*a*, 5*b*, 5*c*, 5*d*). A slightly larger dimension of the freeze-etched fibril could be attributed to the thickness of the replica.

From these observations, it was concluded that the replica elevation corresponds to the light band or a_1-b_2 region, and the depression to the hole region or c_2-d region of negatively or positively stained fibrils, respectively.

In some fibrils, particularly unfixed ones, component filaments could be seen within the fibrils. These filaments were often twisted, with varying pitch lengths ranging from 2 700 Å to 4 800 Å (Figs. 2*a*, 2*b*, 3*b*, 3*a*, 3*b*, 3*c*). These twisted fibrils simultaneous showed regular transverse striations (Figs. 2*a*, 2*b*, 3*b*). In negatively or positively stained fibrils, there was some suggestion of twisted internal component filaments (Fig. 5*e*) but artefact due to teasing could not be ruled out.

DISCUSSION

The surface of the freeze-cleaved dermal collagen as replicated with fine-grain platinum reproduced "true-to-the-nature" ultrastructure in three dimensions. By this new method, the dermal collagen fibrils were shown to have an occasional weaving pattern within the same island, a ground substance surface coat, alternating bands of elevation and depression, and coiled component filaments within the individual fibrils.

These features of dermal collagen could not be elucidated by conventional thin section electron microscopy or by a simple shadow-casting technique, whose resolution was not particularly good. For example, in thin section, dermal collagen fibrils in the same island run parallel to each other (5), probably because within the thickness of 400–600 Å of thin sections all fibrils cut tend to appear similar regardless of their three-dimensional course over the entire length of the fibrils. The lateral borders of the dermal collagen fibrils appear straight without periodic elevations and depressions (5).

According to the model of Hodge & Schmitt (8), Hodge (10), and Hodge & Petruska (9), the hole region, which was shown in this study to be depressed and approximately to cover the c_2-d intraband region, corresponds to a gap or a hole where 4/5 of the unit tropocollagen macromolecules are present and 1/5 are missing. One may assume that a collagen fibril, as a bundle of unit tropocollagen macromolecules, is narrower in diameter in this region than in the overlap region. It is interesting that the same c_2-d band region of the native collagen fibrils is susceptible to electron beam damage (1).

It is currently believed that three tropocollagen units, each twisted around itself in a left-handed helix, twists again in an equilateral spatial relationship around the common major axis, forming a long right-handed helix of about 28.6 Å repeat (2). Such a "coiled coil" structure thus produced represents a tropocollagen macromolecule. When heavily polymerized, these tropocollagen macromolecules produce a protofilament of a mature collagen fibril which was demonstrated in the present study to be twisted. It is possible that the coiled nature of tropocollagen induces larger macromolecules to coil again around each other at a higher organizational level, to produce such large twisted strands as are observed in the present study. Grassmann et al. (3) showed helical tactoids in human dermal collagen after physical and/or chemical isolation procedures. In low magnification pictures of a shadow-cast preparation, however, it is difficult to judge whether such a coiled structure represents an aggregation of two or three mature fibrils or is an internal structure of a single fibril.

Fig. 5. (*a*) A high magnification of replica of fixed collagen fibrils demonstrate alternating elevations (*e*) and depressions (*d*) of about equal intervals. × 117 000. (*b*) As shown in Fig. 4*b*, a negatively (partially positively) stained native collagen fibril shows elevated and depressed (*arrows*) areas. The depressed area contains c_2-d bands. When matched with the replica of 5*a*, the depressed c_2-d region and the elevated a_1-b region of relatively well stained fibrils correspond well in dimension to the depressed and elevated regions of the replica. × 112 500. (*c-f*) The elevated (*e*) and depressed (*d*) zones of a replica of a collagen fibril (5*c*) match well with the light overlap zone (*o*) and the dense hole zone (*h*) of negatively stained native collagen (5*d*). This negatively stained fibril (5*d*) is matched with fibrils which show some stained bands (5*e*) and then the latter in turn with very well stained fibrils (5*f*). From this comparison, it becomes apparent that the elevated zone of the replica roughly corresponds to the a_1-b_2 region of the native collagen and the depressed zone to the c_1-d region of the native collagen fibril. All from the skin of the palm. 5*c*: × 117 000. Others: × 112 500.

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