

UNEVEN NATURAL CROSS-LINKING IN HIDE FIBRILS*

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Abstract

The techniques of differential scanning calorimetry and electron microscopy were combined to show how biochemical treatments can alter the cross-sections of collagen fibrils in calfskin. The denatured cores that were previously shown to develop in the fibrils at 65°C could be removed by digestion with trypsin, which left hollow fibrils as empty sheaths. This will permit manipulation of fibril cross-sections to prepare leather with radically novel textural properties.

Introduction

The fundamental structural element of animal hides used in leather is a fibril about 100 nm thick composed mostly of collagen. Most leathermaking processes involve changing the packing of these fibrils and filling the spaces among them. Although crosslinking of collagen in the fibrils has been studied extensively, little is known about how the physical substructure of the fibrils of hide is affected by tanning and other leathermaking processes.

The cross section of a typical fibril in normal calf skin is contrasted in Fig. 1 (c) with that found (a) in a dermatosparactic mutant with stretchy skin⁽¹⁾. This mutant lacks the enzyme procollagen N-peptidase, resulting in the persistence of pro-N-collagen even in the mature skin. The "cauliflower"-shaped cross sections in Fig. 1a might yield leather with unique properties (desirable or otherwise). They are in some way due to the characteristic of this mutant of failing to completely process collagen after secretion of the protein, so that portions of the propeptide persist in the fibrils. Manufacturers of synthetic fibers go to considerable lengths to achieve novel profiles such as hollow fibers or fibers that stain differentially across their sections. The studies presented here relate to the possibility of preparing leather containing collagen fibril cross-sections with unusual shapes or differential reactivity.

Methods

Skin from the posterior dorsal region of 6-mo calf or of mature (18 mo) steer was frozen within 2 hours of slaughter and stored at -35°C. The reticular dermis was isolated by dissection while the specimen was kept partially frozen, and the sample was kept on ice for less than 4 hours thereafter before the thermal treatment in a stirred water bath with a programmed scanning temperature controller. Specimens were heated at 1C°/minute to various temperatures, quenched on solid carbon dioxide, and then treated for 4 hours at 37°C with 0.1 mg/ml trypsin (Sigma Chemical Corp. No. T8642)** or 0.25 enzymic units/ml chondroitinase ABC (Sigma Chemical Corp. No. C3509)** in 0.1 M phosphate buffer at pH 7.4. After having been quenched with 0.27 mg/ml phenylmethylsulfonyl fluoride

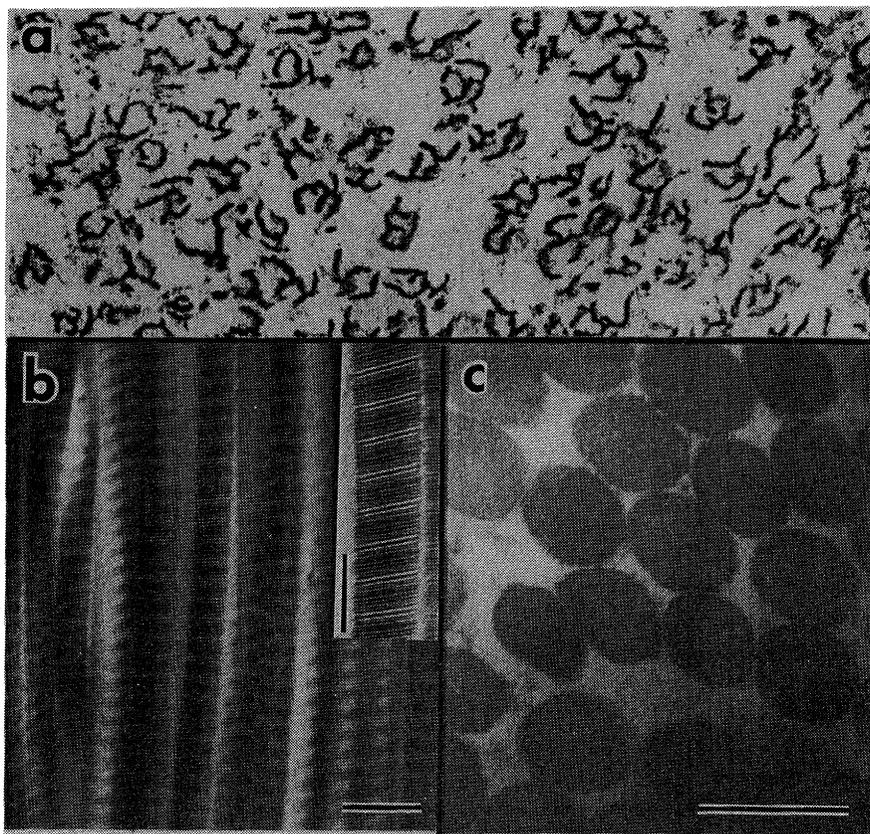


FIG. 1. — Hide collagen fibrils. (a) Cross-sections of dermatosparactic fibrils (Ref. 1***); (b,c) normal fibrils. Bars, 200 nm. Inset: bar, 100 nm.

(trypsin treatment only) the fragments from the digestion were removed by extraction with water at 4°C for 26 hours before being fixed for electron microscopy.

To observe the structure of the collagen residue heated to any certain temperature, we interrupted the scan at that temperature, quickly removed the sample and quenched it with solid carbon dioxide, chopped it with a scalpel, and dropped the fragments into 2.5 percent glutaraldehyde in 0.1 M sodium cacodylate, with 1 percent tannic acid, at pH 7.4, and held them for 18 hours at 22°C. After having been rinsed in this buffer and in water, they were stained *en bloc* with 1 percent aqueous uranyl acetate, placed in a water-soluble melamine resin, Nanoplast™ (Polysciences, Inc., Warrington, PA 18976)**, and cured in a desiccator at 40°C for 48 hours and then at 60°C for 48 hours. When Spurr's epoxy was used for embedding, thin sections of thermally denatured samples sank in the trough of the diamond knife during microtomy, but the melamine resin⁽⁴⁾ yielded sections with clear structures. Micrographs of 60-nm sections were obtained with a Zeiss EM10B electron microscope operated at 60 kV.

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Results and Discussion

Thermograms of collagen from reticular dermis of mature cattle have been published earlier⁽⁵⁾. In calfskin three endotherms are apparent (Fig. 2), with peaks at 56.5°C, 63°C, and ca. 75°C (pronounced shoulder), respectively⁽⁶⁾. The first peak was not observed in the hides of mature cattle⁽⁵⁾; the others were. The third was sensitive to low pH⁽⁵⁾ and to reduction of the various lysine and allysine condensation products with borohydride⁽⁶⁾. The second endotherm persisted as a sharp peak after various treatments including borohydride reduction⁽⁶⁾, aging, tanning (which caused the temperature at the peak to increase), and acid or base, which caused it to decrease⁽⁵⁾.

As shown earlier⁽⁶⁾ the thermogram of natural dermis was represented by the sum of three Gaussian functions well enough to allow us to determine empirically the contribution of each component to the total enthalpy. We argued that each of the three contributions to the enthalpy is proportional to the relative mass of the corresponding collagen population, either 13 percent, 47 percent, or 40 percent, in increasing order of denaturation temperature⁽⁶⁾. We also showed that there was no shrinkage or loss of birefringence until the tissue had been heated to 68°C, when, according to the area under the thermogram, 56 percent of the collagen had already been denatured in the native dermis and from 63 percent to 86 percent in the reduced tissue, varying from sample to sample.

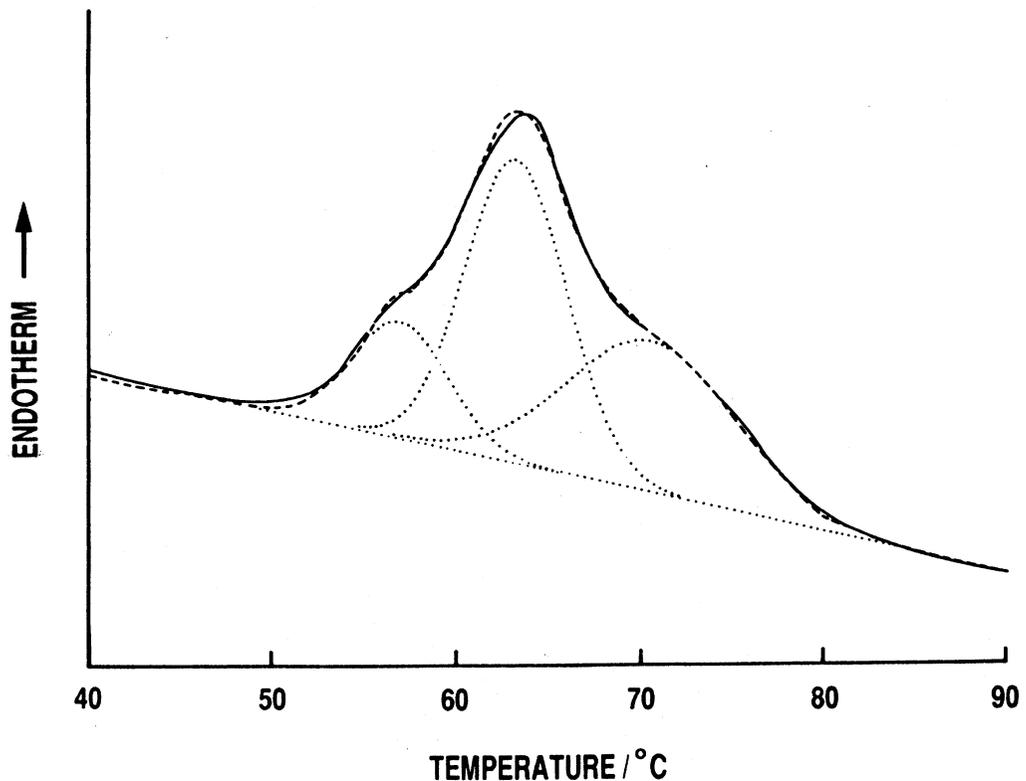


FIG. 2. — Endotherm of collagen in calf dermis (—), showing also three Gaussian components (· · ·) and their resultant (---). The whole ordinate spans 0.2 mW (Ref. 6***).

Embedded in melamine resin and stained with tannic acid-uranyl acetate as described in METHODS, the unheated fibrils in transverse section appeared against a light background and had electron-dense interiors (Fig. 1c); the longitudinal sections (Fig. 1b) had positively stained Schmitt-Gross bands⁽⁹⁾. As can be seen in Figs. 1b and 1c, the edges and bands of the fibrils were clearly resolved. Any changes that might have occurred when the dermis was heated through the first peak to 59°C were not evident by direct examination in the electron microscope.

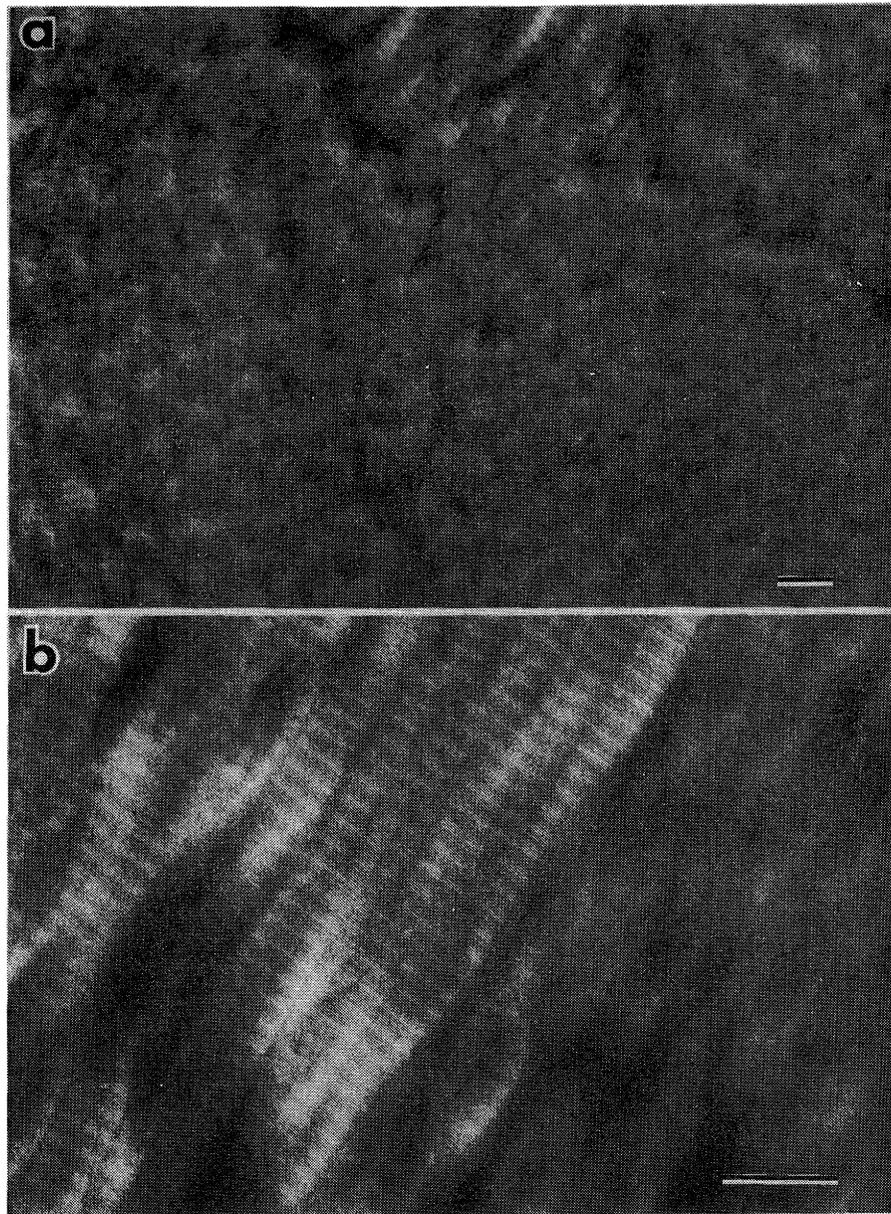


FIG. 3. — Collagen fibrils in heated calf-skin dermis. (a) Transverse sections heated at 1.25°C/min to 63°C; (b) heated at 1.25°C/min to 65°C. Bars, 200 nm.

After heating through the second peak, however, to 63°-65°C, electron-dense areas at the centers appeared in the transverse sections (Fig. 3a). As shown earlier⁽⁶⁾ these cores coincide in their locations with dark streaks on the axes of the longitudinal sections of the still cross-banded fibrils (Fig. 3b). Fig. 3 shows then that the fibrils in the sample that had not yet undergone thermal shrinkage, but had been 55.6 percent denatured (area under the DSC curve in Fig. 2 up to 65°C), had sheaths with obvious intermolecular order (as inferred from the Schmitt-Gross bands) still preserved. Evidence for denaturation of the sheaths along the lengths of the fibrils appeared first in the micrographs after heating to 68°C, at which the fibers had just become isotropic (Fig. 2) and the sample had begun to shrink. The sheaths, like the cores, appeared to be transformed into a dark-staining amorphous material.

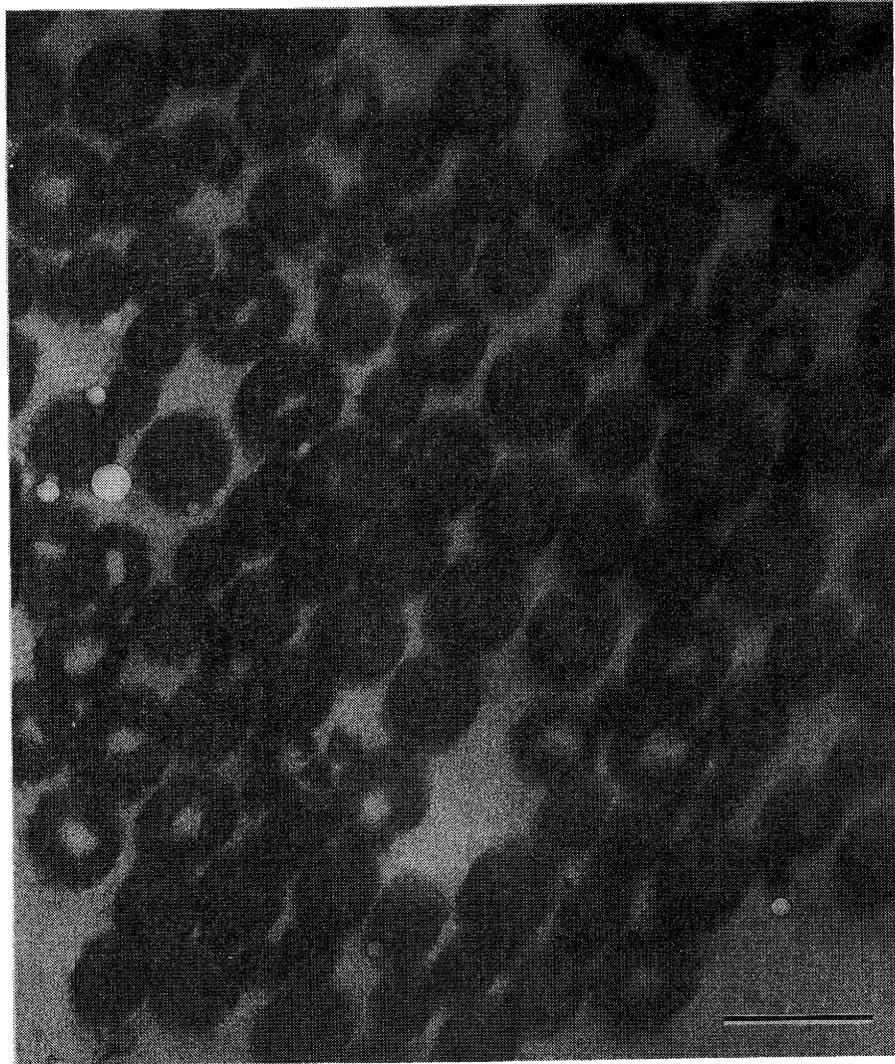


FIG. 4. — Collagen fibrils in calf-skin reticular dermis heated at 1.0C/min to 68°C and then treated with trypsin. Bar, 200 nm.

To determine whether the dark-staining cores were denatured collagen we treated, with trypsin, fibrils heated to various temperatures on the denaturation range shown in Fig. 2, and then examined them with the electron microscope. The sample heated to 68°C is shown in Fig. 4. It is clear that heating and treatment with the enzyme resulted in diminished electron density at the centers of the fibrils. Some fibrils had clearly defined empty-looking cores, as though the centers had been digested away by the trypsin. Unheated fibrils were not apparently altered by the trypsin. It is well known that trypsin does not attack native collagen (or else it could not be used as a bate), but does degrade denatured collagen⁽¹⁰⁾. In addition the dark-staining material has been removed by the trypsin treatment. When chondroitinase ABC was substituted for trypsin, however, the dark cores and background material remained, showing that they are neither dermatan sulfate, chondroitin sulfate, nor hyaluronate, the usual polyanions found in skin.

These results may have implications for tanning or for devising new leathermaking procedures. We have already reported that natural crosslinks are unevenly distributed across hide collagen fibrils, giving a sheath-core structure⁽⁶⁾. Fig. 2 shows that the cores can melt without causing shrinkage. Only when the more stable sheaths melt, however, at temperatures above 68°C, does the sample shrink. These results suggest that, for stability of the leather as a whole, it might be necessary only to add synthetic crosslinks to the sheaths. On the other hand, evaluating a tanning agent of high-molecular weight by measuring the shrinkage temperature might give insufficient information about the general effects of the tan, since it might tan the sheaths alone and raise T_s , even while the cores would still melt below T_s . The shrinkage behavior alone would not be sufficient to evaluate such tans. Further, the uneven distribution could affect the estimation of the number of crosslinks by measurements of thermal tension⁽¹¹⁾ or swelling.

Conclusions

The fibrils of bovine hide have a sheath-core structure that can be developed by heating to temperatures just below the shrinkage temperature. By appropriate enzymic treatment, such as with trypsin, the cores can be selectively dissolved away, leaving hollow collagen fibrils that might impart unusual softness or dyeing properties to leather made from the hide.

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