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Cover Page Footnote

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A SIMPLE METHOD FOR ULTRASTRUCTURAL EVALUATION OF FRAGILE COLLAGENOUS BIOMATERIALS.

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Abstract

The ultrastructural characteristics and quality of preservation of collagen fibers was evaluated by cutting dry fibers at room temperature and fracturing frozen dehydrated fibers. The preparations were examined by scanning or transmission electron microscopy.

The surface of dry collagen fibers cut at room temperature appeared compressed. Observations of the internal substructure were distorted. The open face of the fibers were frequently covered with debris. Conversely, when fibers were frozen and then fractured, dehydrated fibers appeared to have undistorted features and were intact around their edges. No contamination across the face of the fiber was apparent and there was no difference in morphology between the nitrogen and Freon frozen dehydrated collagen fibers. Transmission electron microscopy showed that collagen fibers were composed of a subpopulation of transverse and longitudinal collagen fibrils and fibril bundles embedded in a disorganized amorphous collagen matrix.

Freeze fracturing or snapping collagenous biomaterials was shown to be an effective method for retaining the substructure of these dry and very fragile materials. There may be other applications of this technique to the field of biomaterial morphology.

KEY WORDS: Collagenous materials, collagen fibers, collagen fibrils, biomaterials, freeze fracture.

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Introduction

Many biomaterials are extremely delicate and fragile. Examining the inside of a dry whole material by scanning electron microscopy is problematic in that soft and fragile specimens are vulnerable to compressive and shearing forces induced during cutting of the sample. There are currently several different methods which can be used to examine the inside of fragile materials.

Resin embedding provides support to a material so that sections can be cut to the interior point of interest. This is typically done for transmission electron microscopy. A method to remove the resin from thin sections is described by Winborn and Guerrero (1974). These sections can be subsequently examined using the scanning electron microscope. However, the 3-dimensional panorama typical of a whole mount bulk specimen is compromised since a flat uninteresting image is produced. Alternatively, thick paraffin embedded sections can be examined in the scanning electron microscope by removing the wax (Abraham and DeNee, 1973, 1974; Laane, 1974). Such sections show satisfactory retention of surface and internal substructure and produce a 3-dimensional image. A thorough review on the topic of sectioned specimens for examination in the scanning electron microscope is provided by Laane (1976).

Examining the interior of fixed and dehydrated samples, which are frozen and fractured while still in solvent, is still another method which can be used for exposing the inside of a sample. Humphreys and co-workers (1974) suggest transferring ethanol dehydrated specimens to liquid nitrogen, fracturing the

deeply frozen tissue and then critical point drying. Konerding and co-workers (1988) transferred the sample from 100% ethanol to fluid propane gas, in a liquid nitrogen environment, then fractured the sample and critical point dried it. In these examples, the specimen is already fixed, dehydrated and cryoprotected against freezing artifacts. The methods produce excellent preservation of interior substructure.

In the case of rigid dry materials which need not be fixed and dehydrated, viewing the interior involves simply cutting the specimen in half. Wooden stems and dry seeds for example can be cut with a razor blade and examined in the scanning electron microscope (Olsen, 1974). Some dry materials are too flexible to be sectioned at room temperature. When cut in half, the true ultrastructure of these samples may be distorted due to compression and shearing artifacts. For example, cutting ultrathin frozen sections of some polymers produces ultrastructural images that are improved from images obtained from sections cut at room temperature (Attenburrow and Lewis, 1972). Olsen (1974) describes a method for freezing and cutting sections on a microtome for scanning electron microscopic examination. This method provides excellent retention of internal substructure.

In this paper the substructural preservation of reconstituted type I collagen fibers prepared by 2 different methodologies is compared. In this laboratory collagen fibers are used *in vitro* for the support of cell growth (Wasserman et al., 1988; Kato et al., 1990) and *in vivo* for soft tissue replacement (Goldstein et al., 1989; Wasserman et al., 1988, 1989a, b, c). The first method is cutting the dry unfixed materials and the second is fracturing frozen dehydrated materials. Collagen fibers prepared by the above methods were examined using a scanning electron microscope. The interiors of collagen fibers were also examined using a transmission electron microscope. A simple procedure for stabilizing frail materials on scanning electron microscope stubs is also presented.

Materials and Methods

Insoluble collagen obtained from bovine hide corium was used for production of collagen fibers as previously described (Kato et al., 1988, 1989; Wasserman et al., 1989a). In

brief, a room temperature 1% (w/v) collagen dispersion, prepared from bovine hide, was extruded through polyethylene tubing with an inner diameter of 0.28 mm into a 37°C, pH 7.5, fiber formation buffer. After 45 mins in the buffer, the fibers were rinsed in isopropyl alcohol for 4 hrs followed by distilled water for 20 mins. The fibers were then air dried and crosslinked with glutaraldehyde vapor.

The interior of collagen fibers was exposed by deep freezing 1 cm segments of fiber in liquid nitrogen or liquid Freon. Fibers frozen in Freon were then transferred to liquid nitrogen. Using irradiectomy scissors the first and last piece of each segment (which contained ends previously exposed to the atmosphere) were cut (snapped or fractured) away and discarded. Using the irradiectomy scissors each remaining frozen segment of the collagen fiber was fractured in half. By this method each half of the original 1 cm segment had a freshly cleaved top and bottom surface. There could be no confusion as to which half was the freshly cut surface. The segments were transferred to an aluminum foil pouch (in liquid nitrogen). The pouch was transferred to a bell jar in a liquid nitrogen cooled transport device and pumped to a vacuum of 100 mTorr overnight. This minimized frost contamination as the fiber segments came to room temperature. Each fiber segment was then mounted to a glass pipette fragment (or ideally a rectangular metal scaffold if available) which was secured to an aluminum stub with silver paste. In this way, the cut faces of the fibers were stabilized, standing straight up in the air and fully accessible for examination by the electron beam (Figure 1). All fibers were sputter-coated with 40 nm of gold-palladium and examined at 10 kV on an AMRAY 1400 scanning electron microscope equipped with a titanium sublimation pump.

The interiors of a second group of 1 cm collagen fiber segments were exposed by cutting the dry room temperature fibers in half with irradiectomy scissors. These fibers were then mounted as described above and examined on the scanning electron microscope.

A third group of fibers were prepared for transmission electron microscopy according to the method of Scott (1980). Collagen fibers were fixed in 2.5% glutaraldehyde (v/v), 0.3 M MgCl₂ and 0.025 M sodium acetate buffer (w/v), pH 5.7 containing 0.05% quinolinic blue (w/v; Polysciences, Inc., Warrington, PA). The

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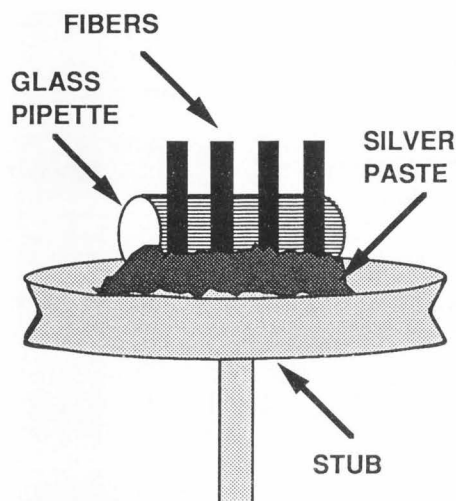


Figure 1. Diagram showing the arrangement of collagen fibers on a stub for examination in the scanning electron microscope. A small fragment of a glass pipette is glued to a stub with silver paste. The collagen fibers are then glued to the pipette so they are standing straight up in the air.

fibers were washed in buffer (fixative solution without stain), distilled water, dehydrated and embedded. Thin sections were double stained in saturated uranyl acetate (aqueous) and 1% sodium tungstate (w/v; aqueous).

Results

Scanning electron microscopic observations of the interior of a dry collagen fiber, cut at room temperature are shown in Figure 2. These micrographs show that the fiber appears compressed (Figure 2a). Some fibers had loose fragments of debris around their edges and upon their surfaces (Figure 2b). Ten fibers were examined and it was difficult to clearly observe the substructure of any fiber in this group.

Scanning electron microscopic images, obtained from dry collagen fibers frozen by immersion in liquid nitrogen and then fractured are shown in Figure 3. The collagen fibers were intact around their edges (Figure 3a, 3d). The fibers were composed of a subpopulation of individual transverse and longitudinal fibrils. These fibrils were also organized into higher order substructures: transverse (Figure 3b) and longitudinal (Figure 3c) collagen fibril bundles. Rarely was there any debris on the face of the fibers. Of the 40 fibers examined by freezing and fracturing, 5

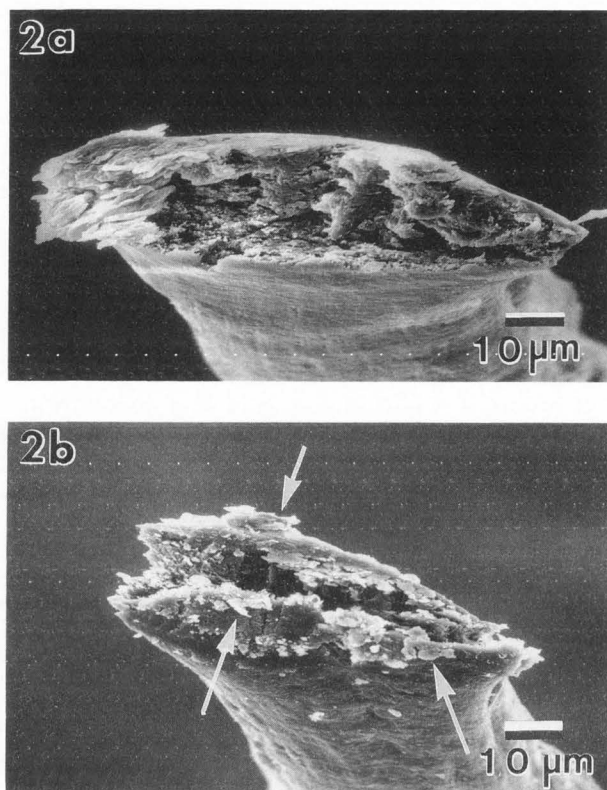


Figure 2. Scanning electron micrographs of dry collagen fibers cut with an iris scissors. (a) Notice that this fiber appears compressed and the face of the fiber is distorted. (b) In this micrograph, it appears that the fiber face is also compressed and covered with debris (arrows).

had cross fractures which resulted in uneven fiber faces. One fiber had a large depression or divot on its surface and cracks were observed along its sides (Figure 3d). There were no observable differences between fibers frozen in liquid nitrogen and Freon.

The interior morphology of frozen dehydrated collagen fibers as seen by scanning electron microscopy was confirmed by transmission electron microscopic observations (Figure 4). The fiber interior contained a subpopulation of individual longitudinal and transverse collagen fibrils and fibril bundles (Figures 4a, b, c, d). These structures were embedded in a less organized matrix of collagen (Figure 4b). Transverse fibrils had a striated appearance characteristic of type I collagen (Figure 4b, 4c). The individual collagen fibrils had an amorphous appearance and assumed a roughly circular or orthogonal shape when viewed in cross section (Figure 4d).

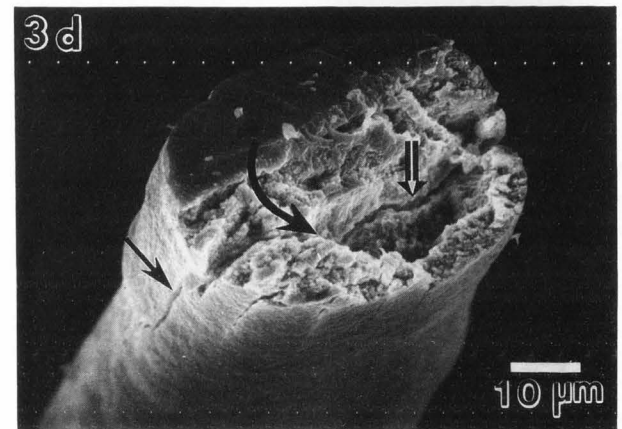
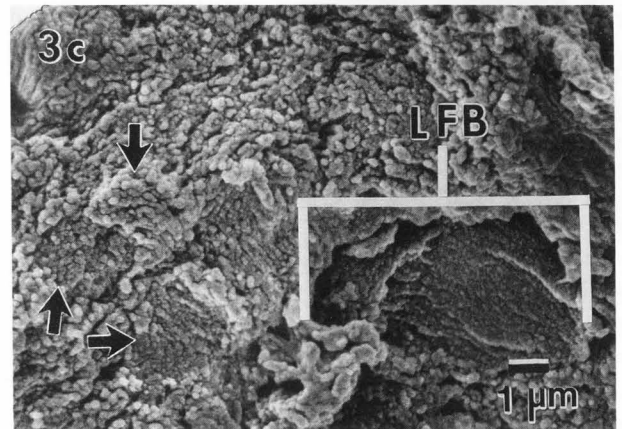
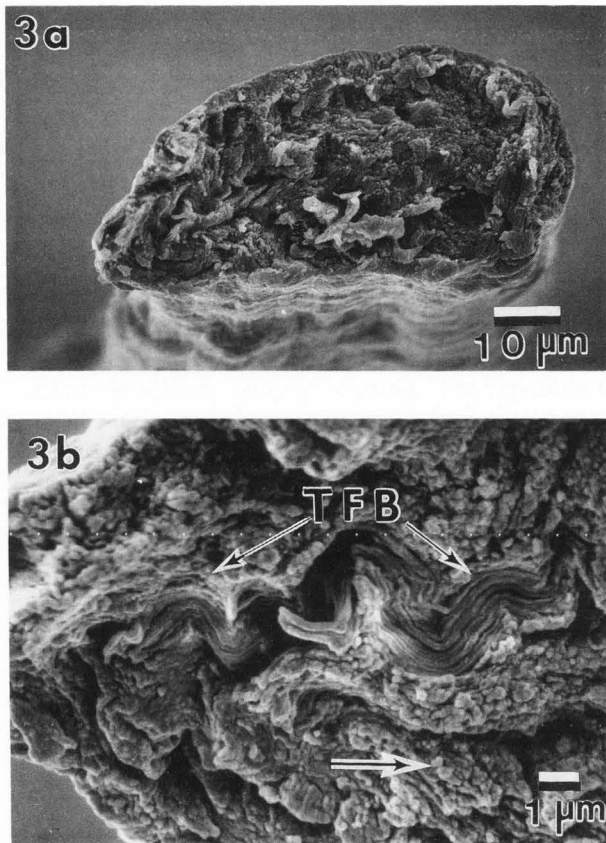


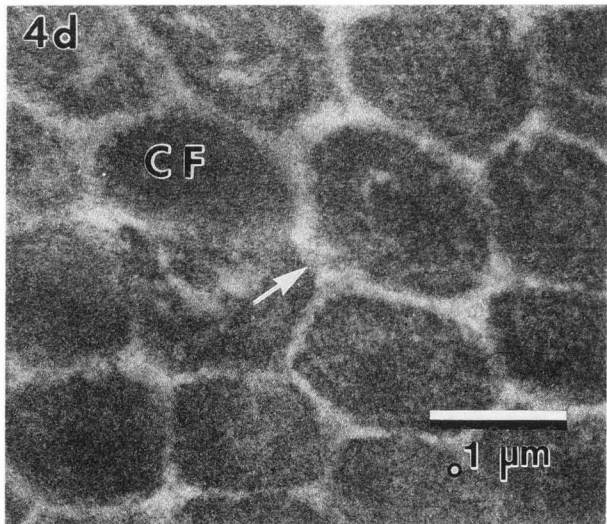
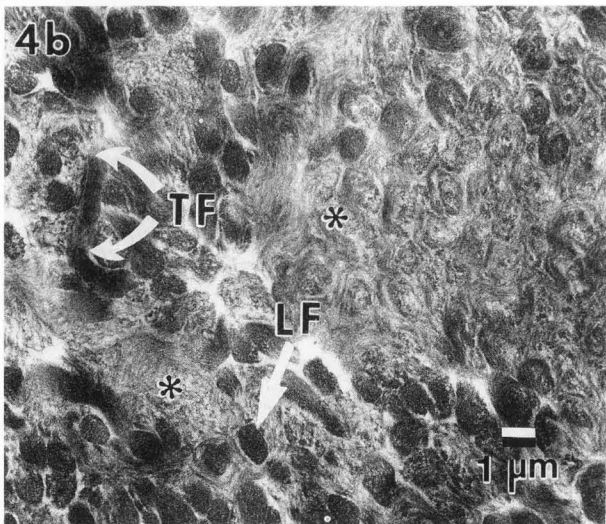
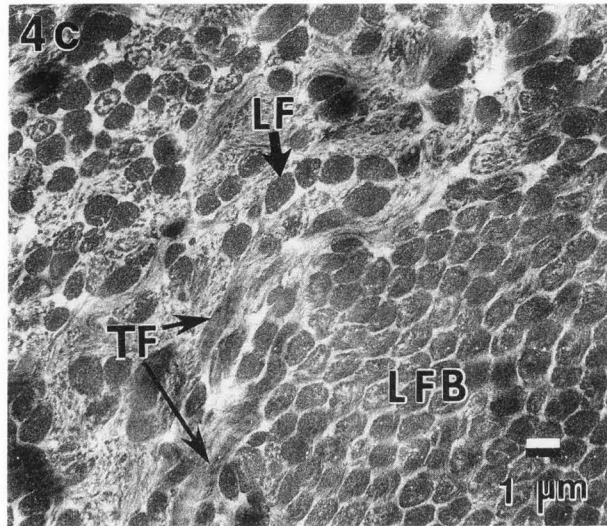
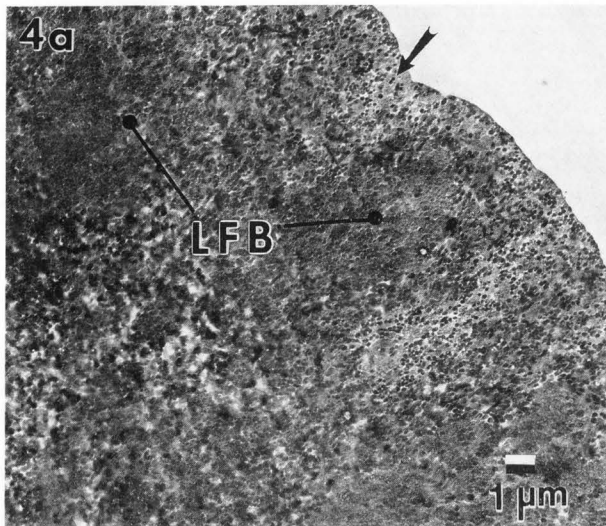
Figure 3. Scanning electron micrographs of dry collagen fibers frozen in liquid nitrogen and fractured with iris scissors. (a) Seen here is the face of a fiber having intact edges. (b) Within this fiber can be seen individual longitudinal collagen fibrils (arrow) and a very large transverse collagen fibril bundle (TFB). Notice that within the bundle are individual transverse fibrils (compare to the longitudinal and transverse fibrils seen by TEM in Figures 4b, 4c and 4d). (c) Seen in this micrograph are longitudinally arranged fibril bundles (LFB and arrows; compare to the LFB seen by TEM in Figure 4a). (d) This collagen fiber has intact and undistorted edges. Notice there is a large cavity in its face (curved arrow) representing a site where some substructural element of the fiber has "popped" out during the fracture. There are also cracks along the sides of the fiber and inside of the cavity (arrows).

Discussion

One of the great virtues of reconstituted collagen is that it can be engineered into a broad spectrum of biomedical devices all having specific applications to biological and

Figure 4. Transmission electron micrographs of dry collagen fibers embedded in resin and thin sectioned to expose the interior. (a) Seen here is an overview of the fiber showing its very delicate fibrillar substructure. Notice the individual collagen fibrils (arrow) which appear as fine dots distributed throughout the fiber interior (Compare to Figures 4b, 4c, and 4c). Longitudinal fibril bundles are also present (LFB; compare to the LFB seen by scanning electron microscopic examination in Figure 3c). (b) This micrograph shows collagen fibrils embedded in a less organized matrix of collagen (asterisk). Notice that there are both longitudinal (LF) and transverse (TF) collagen fibrils present. (c) The corner of a large longitudinal fibril bundle (LFB) is seen in this micrograph. Notice also the individual longitudinal (LF) and transverse (TF) collagen fibrils. (d) Individual collagen fibrils (CF) are featured in this micrograph. Notice they appear to be rather amorphous. There is no distinct delineation where the fibrils end. The matrix of each fibril appears to attenuate at its periphery (arrow).

Collagenous Biomaterials



medical problems. Reconstituted type I bovine collagen fibers are used extensively in this laboratory for growing nerve cells (Wasserman et al., 1988), as a tendon replacement (Goldstein et al., 1989; Wasserman et al., 1988; Wasserman et al., 1989a, b) and as a nerve conduit (Wasserman et al., 1989c). The efficacy of a biomaterial for tissue augmentation and replacement is determined by several parameters.

The biochemical composition of a biomaterial can affect its biocompatibility. The composition and type of collagen used in the biomedical devices prepared in this laboratory is Type I as previously established using amino acid analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Weadock et al., 1984). This collagen has a positive

chemotactic effect on cells involved in wound healing (Kleinman et al., 1981; Dunn and Ebendal, 1978; Grinnell and Bennett, 1982; Thomaseck et al., 1982).

The morphological structure of a biomaterial may also affect material biocompatibility. Epithelial cells grown on a collagen film develop better cell-to-cell contact than cells grown on plastic (Doillon et al., 1986; 1987; 1988). Nerve cells grown on collagen fibers will orient themselves relative to the longitudinal axis of the fibers (Wasserman et al., 1989c) and fibroblast-like cells will align themselves relative to the longitudinal axis of a grooved substrate (Ricci et al., 1984). Because cells respond to the structure of the material they grow on, the physical characteristics of that material should be understood. Collagen

fibers biodegrade when used as an implanted device. Therefore, both the external (surface) and internal structural characteristics may modulate their interactions with cells.

In an effort to visualize the interior of dry collagen fibers they were simply cut with an irradectomy scissors to expose the inside. This resulted in fiber and fibril compression and distortion making an accurate interpretation of the substructure difficult. To circumvent this complication, fibers were first deep frozen in liquid nitrogen or Freon and then snapped into pieces to expose a virgin surface. By cutting the frozen fiber it was possible to prevent compression and distortion of the fiber.

Liquid nitrogen boils or bubbles when it surrounds a sample thereby insulating and producing slow freezing rates. Biological samples require freezing rates above 5-10,000°C per second to prevent ice crystal formation at the ultrastructural level (Bullivant, 1970; Christiansen, 1971; Moor, 1971). However, in the present study, fibers were not wet and there can be no vitreous freezing of dry materials. Eutectic and ice crystal formation do not occur. It appears that freezing dry materials in liquid nitrogen adequately preserves material substructure.

Both transmission and scanning electron microscopic observations showed that collagen fibers contained a subpopulation of transverse and longitudinal fibrils and fibril bundles that were contained within a matrix of less organized collagen. Divots or pits, observed on the fractured interior fiber face, may represent sites where a transverse fibril bundle was "popped out" of the fiber. In a separate set of experiments, similar pits were observed on fiber faces exposed by pulling dry fibers in half. When the opposite half of the pulled fiber was examined, the transverse fibril bundle was identified (Y.P. Kato, personal communication).

Collagen fibril bundles, observed in these studies, may have formed as the result of fibrillogenesis. Conversely, the fibril bundles could have been remnants of undispersed bundles of fibrils originating in the bovine hide corium used for the preparation of the collagen dispersion (Kato et al., 1989). If fibril bundles are an artifact (undispersed fibrils from the corium) it is likely they are a factor limiting the overall biomechanical properties of the fiber since they create a weak point (especially transverse bundles). This may be an important consideration in applications where strength of the implant is of primary concern.

The simple freeze and fracture method described in this paper is effective for preserving the interior substructure of dry collagen fibers. Specimen fixation, dehydration and critical point drying are unnecessary as are microtomes and freeze fracture equipment. This method may be applicable to the scanning electron microscopic study of other soft, fragile and dry material interiors.

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Discussion with Reviewers

C.J. Doillon: In all procedures, osmium tetroxide is not used. Under SEM and TEM, the post fixation by OsO₄ improves the preservation of the ultrastructure and this is particularly important during freezing and freeze-fracture of the specimen. There is an excellent method described by Osatake et al. (1985). This technique is less simple than the one described by Wasserman. However, I have personally used this method which produces excellent results and I believe the method should be mentioned in this paper.

Authors: We routinely use OsO₄ for the preparations of samples for TEM and SEM. There is little doubt that post-fixation in OsO₄ increases the quality of overall fixation and conduction of charge (away from the specimen) during viewing. In addition I typically block stain specimens in 1% uranyl acetate after osmication in order to further fix and reduce charging. For the TEM preparations we used the method of John Scott (1980). This technique which specifically stains for proteoglycans does not employ post-fixation with OsO₄.

As for the SEM studies, the primary goal was to establish a streamlined, inexpensive and simple method for examining the interior of fragile biological specimens. For this reason the usual fixation, osmication and block staining procedure was deliberately omitted. What is unique in our method is that the sample need not be rapidly frozen as long as it is dry.

I concur Dr. Doillon's observations that the method of Osatake et al., (1985) is one which produces excellent results. I too have used this method and have obtained nice results. For that matter, I have also employed traditional freeze fracture methods (low angle rotary shadowing) and complementary replica formation to examine biological specimens for TEM (which is a viable alternative to viewing the inside of a fragile specimen by a method other than thin sectioning of plastic embedded samples). All of these methods although valuable in terms of the information they supply are more complicated (as pointed out by Dr. Doillon) than the method which we present here.

J.L. Ricci: Using this method, fibers will be predisposed to fail at weak points (stress risers) in the material. The transverse fiber bundles may act as such points. Have the authors noted a greater incidence of exposed transverse fiber bundles in the snapped samples versus the TEM sections (which would represent a random sample)?

Authors: The fractured samples observed by scanning electron microscopy should represent just as much of a random sampling of the transverse fiber bundles as the transmission electron microscopy. The point of examination by the fracture method is selected by the investigator. That point is then exposed and examined. If there is a transverse fiber bundle there it will be exposed. Similarly, when sampling for transmission electron microscopy, The investigator selects the area of the fiber to be examined and thin sections are cut for transmission electron microscope examination. We have seen equal numbers of transverse fibril bundles by both methods. However, it is this authors feeling that the transverse fibril bundles do represent a weak point in the fiber. It is possible that alignment of collagen fibrils can be achieved by extraneous "factors" imposed on the fibers during the process of polymerization. Again, this is the topic of a separate investigation.

J.L. Ricci: The authors question whether the observed fibril bundles originated from fibrillogenesis or from undispersed elements of the bovine hide corium. Have the authors conducted TEM or SEM examination (using the new technique) of unreacted collagen dispersion (prior to extrusion into buffer)? If undispersed collagen fibrils are present they should be noticeable among the amorphous dispersed collagen.

Authors: This is a very good question. The experiment should reveal some valuable information. It is part of a thesis currently being performed in our laboratory. We hope these results will be presented at the 1991, Biomaterials Session.