Ultrastructural Changes in the Architecture of Collagen in the Human Cervix Treated With Urea

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Injection of a solution of 30% urea in acetate buffer at pH 4.0 into the stroma of the uterine cervix results in a marked change in the mechanical properties of the tissue with subsequent easy dilatation. This study analyzes the changes in the collagenous matrix of the cervix by ultrastructural and biochemical methods. Collagen fibrils in urea-treated sections of the cervix are swollen and unravelled, showing spiral configuration of subunits in both cross-sections and longitudinal sections. The regular localization of ruthenium-red-positive material is absent in urea-treated tissues. Chemical analysis of incubated cervical tissues shows a reduction of the total glycosaminoglycans and of dermatan sulfate with release of the latter into the medium. It is suggested that urea dissociates intercollagen linkages by solubilizing a certain glycosaminoglycan, possibly dermatan sulfate. After this solubilization the collagen fibril is prompted to unwind, resolving the collagen microfibrils, which appear to be organized in a spiral fashion. (Am J Pathol 1980, 99:525-538)

IN A RECENT paper, it was demonstrated that an injection of 30% urea solution in acetate buffer at pH 4.0 into the stroma of the uterine cervix strikingly softened the tissue, allowing easy surgical dilatation.[1] This effect became manifest 30 minutes after the injection and lasted for a few hours.

While urea has long been recognized in protein and, mainly, collagen chemistry to dissociate the helical structure of collagen by interfering with hydrophobic linkages and H-bonds,[2,3] other mechanisms of the effect of urea could be involved within a complex structure like cervical tissue. The importance of collagen–glycosaminoglycans interaction for the mechanical characteristics of certain tissues has been stressed by several authors.[4-6] Gelman and Blackwell [7] showed that the structural stability of collagen is increased by interaction with certain glycosaminoglycans. It has also been suggested that such a reaction could be an essential step in the formation of collagen fibrils.[8] A recent study by Lillie et al [9] presented evidence for a helical organization of the collagen microfibrils. This helical superstructure of collagen fibrils was demonstrated in human dermis treated with the urea-quanidine type of lyotropic agents. The authors did

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not examine possible changes in the polysaccharide moiety of the collagen hierarchic structure, which were quite prominent in our studies of the cervix given injections of urea. An additional study by Ruggeri et al [10] confirmed helicoid microfibrillar architecture in dermis but also revealed a straight relationship in rat rib and bovine nasal septum and other tissues. These investigators also did not examine a possible structural relationship between the hierarchy of collagen structure and glycosaminoglycans (GAGs).

This study presents the ultrastructural observations of the effect of urea on collagen-polysaccharide structure and shows that helical orgainization of collagen fibrils is also present in this tissue. Changes in ruthenium-red–stained structures after urea treatment are also documented, and the results, supported by direct chemical analysis of GAG extractibility, implicate their involvement in the unwinding of collagen superstructure and infrastructure.

Materials and Methods

Morphology

Wedges of fresh cervical tissue were obtained from patients undergoing hysterectomy. The epithelium was removed as soon as the tissue was obtained from the operating room. The tissue was placed in a glass vessel submerged in an ice bath and taken to the laboratory for processing. Wedges were cut into four approximately equal parts, each measuring about 10 x 7 x 5 mm. One piece was cored twice with a modified Jahmshidi liver punch 1 mm in diameter. The cores were fixed in Karnovsky's solution [11] for study by electron microscopy. The remaining tissue was immediately frozen. The three other pieces were cut and diced by hand with a stainless steel razor blade into pieces as small as possible. The average size approximated 2 cu mm. The diced tissue of each sample was placed in a 50-ml flask. One flask contained 35 ml of 30% urea (w/v) in a 0.15 M sodium acetate buffer, pH 4.8. A second flask contained only the 0.15 M sodium acetate buffer, pH 4.8. A third flask contained only the 0.15 M sodium acetate buffer, at pH 7.2. The flasks were lightly stoppered and incubated in a humidified incubator under continuous shaking for 2 hours at 37.5 C.

After incubation, two small pieces of tissue from each flask were removed and fixed in Karnovsky's solution for examination by electron microscopy. Additional pieces were selected and placed in Karnovsky's with ruthenium red. For these samples the succeeding buffer wash and postfixation osmium totroxide also contained ruthenium red, according to the method of Highton et al.[12] The solutions and diced tissue were gently swirled and poured into 50-ml plastic centrifuge tubes. An additional 5 ml of the appropriate solution was used to wash the flasks, and this was added to each centrifuge tube. The suspensions were centrifuged for 5 minutes at 2000 rpm.

The supernatant of each tube was decanted into a clean plastic bottle. Supernatants and sediments were then frozen immediately in liquid nitrogen and placed in the deep freeze for glycosaminoglycan studies.

The 1-mm cores fixed from fresh cervical tissue and the samples fixed from the incubations were washed in cacodylate buffer after several days in Karnovsky's fixative and postfixed in isotonic OsO_4. Following dehydration in graded ethanols, the tissues were embedded in Epon 812. When cutting thin sections, care was taken to examine the outer

areas of the tissue block because the extent of penetration of the solutions, particularly the urea, was not known.

Biochemical Analysis

The frozen samples of cervical tissue and supernatants of incubated tissue fragments were assayed for glycosaminoglycans as previously described by Shetlar et al.[13]

The tissues were thawed and rinsed with distilled water, dried on filter paper, weighed, and placed in screw-capped test tubes. The tissue was digested at 65 C for 16 hours with papain solution made in acetate buffer containing cysteine, as originally described by Mier and Wood.[14]

The papain digests were treated with trichloracetic acid solution to a concentration of 5% and allowed to set for several hours at 4 C. The precipitate was removed by centrifugation and the supernatant solutions dialyzed against distilled water for 72 hours at 4 C with frequent changes of water. The glycosaminoglycans were precipitated from these solutions with 4 volumes of ethanol containing 1% potassium acetate and 1% acetic acid. The precipitates were again removed by centrifugation and redissolved in distilled water.

The supernatant solutions of the incubated tissue were subjected to cellulose acetate electrophoresis using electrolyte consisting of strips of calcium acetate (0.3 M, pH 7.25), zinc sulfate (0.2 M, pH 5.1) and barbital buffers. Electrophoretic strips were developed by staining with 1% alcian blue in 5% acetic acid and quantitating the bands of glycosaminoglycans in a Beckman Microzone densitometer.

Uronic acid determinations were made on the glycosaminoglycan preparations by the method of Bitter and Muir.[15]

Results

Transmission Electron Microscopy

The effect of 30% urea is shown in Figures 1, 3, 4, 8, and 9. Collagen fibrils, cut in cross-section, appear swollen and unraveled, with individual subunits resolved. Those affected by the urea vary in diameter, the largest of which measures up to 2000 Å. Some sections of fibrils have apparently been unaffected and may be compared with those showing the effect (arrows). A clearer comparison may be made with an unincubated control section in which the collagen fibrils measure 500 Å in diameter (Figure 2). The control tissues from 0.15 M sodium acetate buffers, pH 4.8 and 7.2, demonstrated identical collagen structure to the unincubated controls (Figure 7).

Often a spiral configuration of the collagen fibril subunits can be observed, whether in cross-section or long section (Figure 1, arrows; Figure 3 and Figure 8). Some longitudinal sections show normal-sized banded segments, the ends of which are sprayed as if being unwound (Figures 3 and 4).

Control tissue samples processed with ruthenium red demonstrate periodic localization along longitudinal sections of the collagen (Figure 5, arrows) and a peripheral coating of the ruthenium-red–positive material in cross-section (Figure 6). In the former case, the ruthenium red is in globu-

Table 1—Effect of pH and Urea on the Content of Glycosaminoglycans in Cervical Tissue

Treatment	Uronic acid (mg/g wet weight)		
	Total	Dermatan sulfate	Hyaluronic acid
Control, intact	0.84	0.55	0.16
Control, pH 4.8	0.74	0.53	0.19
Urea, pH 4.8	0.41	0.19	0.22

Average values of two or three determinations are presented.

lar form. Both of the 0.15 M sodium acetate controls show the same kind of distribution (Figure 7).

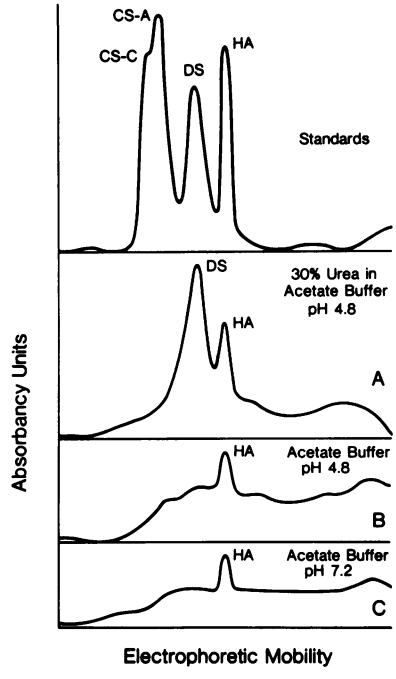
The urea-treated tissues processed with ruthenium red show a loss of the ruthenium-red–stained material about the collagen whether the latter are observed in cross-section (Figure 8) or long section (Figure 9). It is also interesting to note that the globular forms of ruthenium red are absent even in collagen segments, which appear of normal size. Of further interest is the absence of the ruthenium-red–stained spiral configurations of the smaller fibrils in segments that have unwound.

Glycosaminoglycan Assay

Direct assay for the content of glycosaminoglycans and two of their major components, ie, dermatan sulfate and hyaluronic acid, in the cervical tissue before and after incubation in the given media (Table 1) shows that acidic urea reduces the content of both total GAG and dermatan sulfate. There was no effect on the content of hyaluronic acid in the tissue. The evidence for the dissociation and solubilization of dermatan sulfate from the urea incubated cervical tissue is presented in Figure 10. Large quantities of dermatan sulfate were found in the supernate from only urea-incubated tissue. Treatment of the cervix with acetate buffer of pH 4.8 or 7.2 did not extract dermatan sulfate. Only hyaluronic acid was detected in the supernatants of these controls.

Discussion

Incubation of slices of cervical tissue in acidic urea results in two major changes in this collagen-rich tissue: 1) dissociation of collagen fibrils and 2) solubilization of glycosaminoglycans, seen in intact tissue as ruthenium-red–positive globules aligned along collagen fibrils. The appearance of the fibrils of collagen after acidic urea treatment indicates that their subunits are linked together by rather weak cohesive forces, such as hydrogen bonds, ionic forces, or hydrophobic contacts. No covalent link should be involved, as this is resistant to urea or acid cleavage. Only aligned micro-



TEXT-FIGURE 1—Densitometer tracings of glycosaminoglycans electrophoretic mobilities on cellulose acetate strips from the supernatants of the incubation solutions. Solution A is the 30% urea solution, and Solutions B and C the acetate buffer controls. Dermatan sulfate appears only in the urea solution.

fibrils forming the individual fibril unit of collagen show the characteristic repetitive structural pattern (see Figures 3 and 4).

When the urea-induced unwinding of the collagen fibril is just beginning and is viewed from the cross-sectional aspect, a helical, spiral arrangement of microfibrils is clearly evident. It has the same whorl-like appearance (see Figure 8) as that described by Lillie et al [9] for urea or guanidine-treated dermis. In the more advanced effect the spiral appearance becomes lost. In urea-treated tissue, unstained, the spiral forms are much less pronounced than when stained only by ruthenium red. This supports internalization of glycosaminoglycans for structural integrity of the collagen fibril.

There appears to be an association between the solubilization of dermatan sulfate and the loss of ruthenium red globules after urea treatment. No interfibrillar or peripheral fibrillar ruthenium red staining was observed in the urea-treated tissues (see Figure 9). The fact that no ruthenium red staining was observed along the collagen fibrils in the urea-treated tissues, even on segments that had not yet unwound, speaks for the mediation of the GAGs (probably dermatan sulfate) in the hierarchy of the microstructure of collagen. Specifically, this means that within fascicles of colla-

gen, the interfibrillar linkages are mediated by a GAG, much as the microfibrils are bound to structure the basic fibrillar unit of collagen.

The dramatic softening of cervical tissue may well be due to an initial loss of fibrillar intercoiling within the fascicle, following loss of a GAG linkage, and later to an unwinding of the fibril itself.

Therefore, we postulate that urea dissociates (and probably solubilizes) a certain class of glycosaminoglycan (possibly dermatan sulfate), first, from the intercollagen linkage and, second, within the collagen fibril to resolve the collagen microfibrils. This linkage is essential to the structural stability of the collagen fascicle and for the proper alignment of microfilaments into a fibril. Since the effect of urea is not uniform along the collagen fibril length, we could assume variability in the density of cohesive forces within the longitudinal axis of the fibril. The same conclusion was drawn by Lillie et al.[9]

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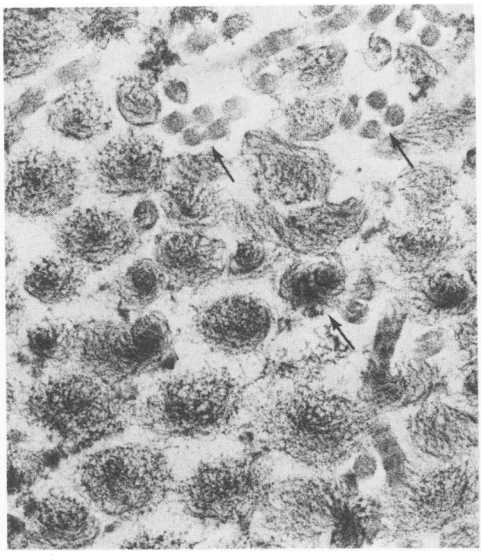
Figure 1—Cross-sectional views of collagen fibrils treated with 30% urea. Largest diameter measures 2000 Å. (×50,000)

Figure 2—Unincubated control cervical tissue. Collagen fibrils are viewed in cross-section and average 500 Å in diameter. (×50,000)

Figure 3—Long sections of urea treated collagen. Unraveling of fibrils (*arrows*) show helical organization. (×50,000)

Figure 4—Cross- and longitudinal sections of urea-treated collagen. Some fibrils show normal-sized segments with both ends unraveled and sprayed. (×50,000)



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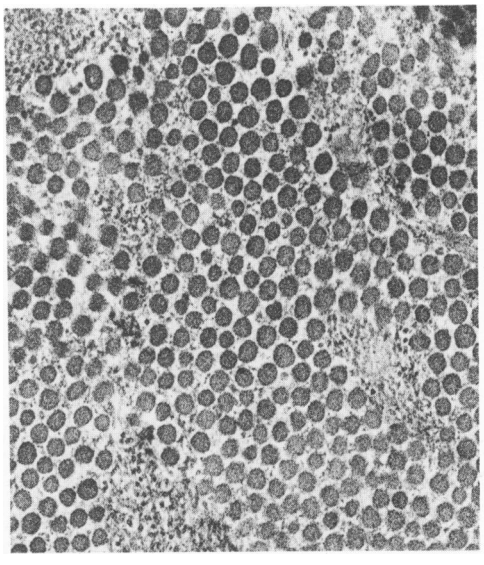

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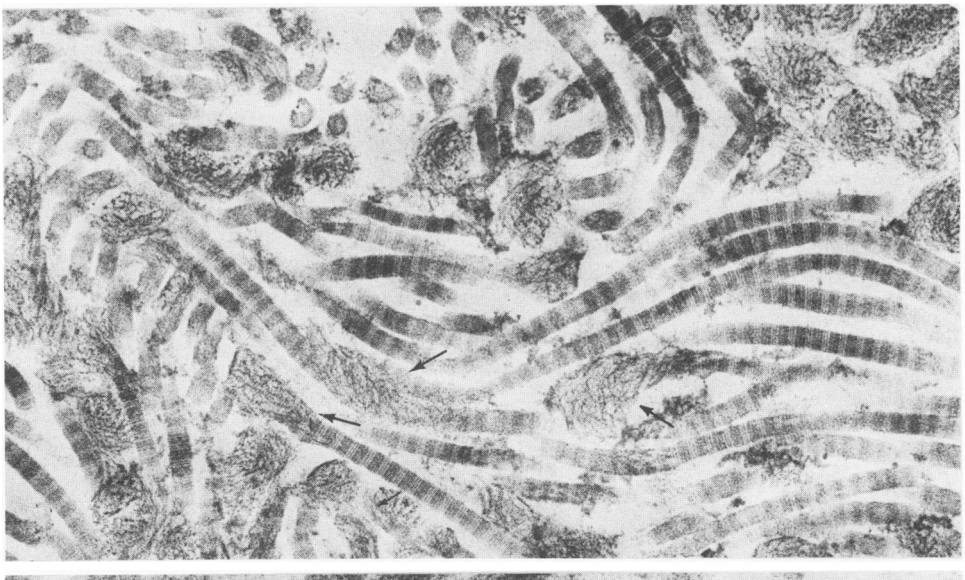

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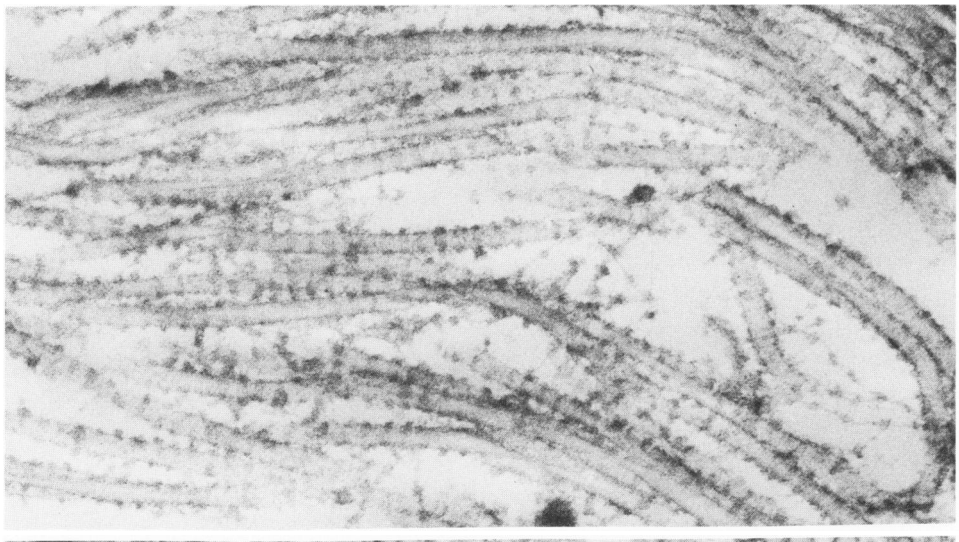
Figure 5—Control tissue, immediately fixed without incubation. Stained only with ruthenium red. Note the periodic localization of positive staining along the fibrils. (×50,000)

Figure 6—Cross-sections of fibrils immediately fixed without incubation. Ruthenium-red-stained only. Note its peripheral distribution. (×50,000)

Figure 7—Long sections of ruthenium-red-stained collagen fibrils from control incubation, acetate buffer, pH 4.8. Ruthenium red deposits persist periodically along fibrils. No unraveling occurs. (×50,000)



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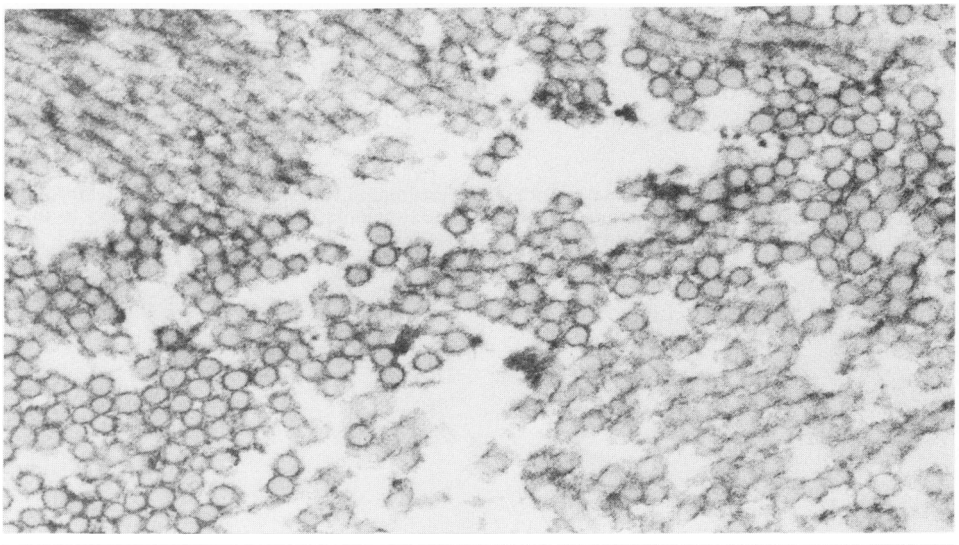

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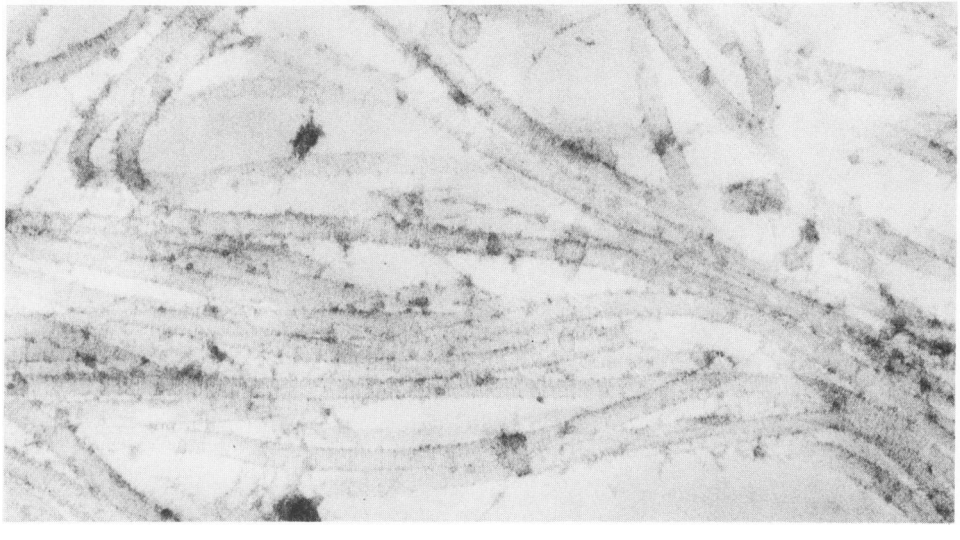

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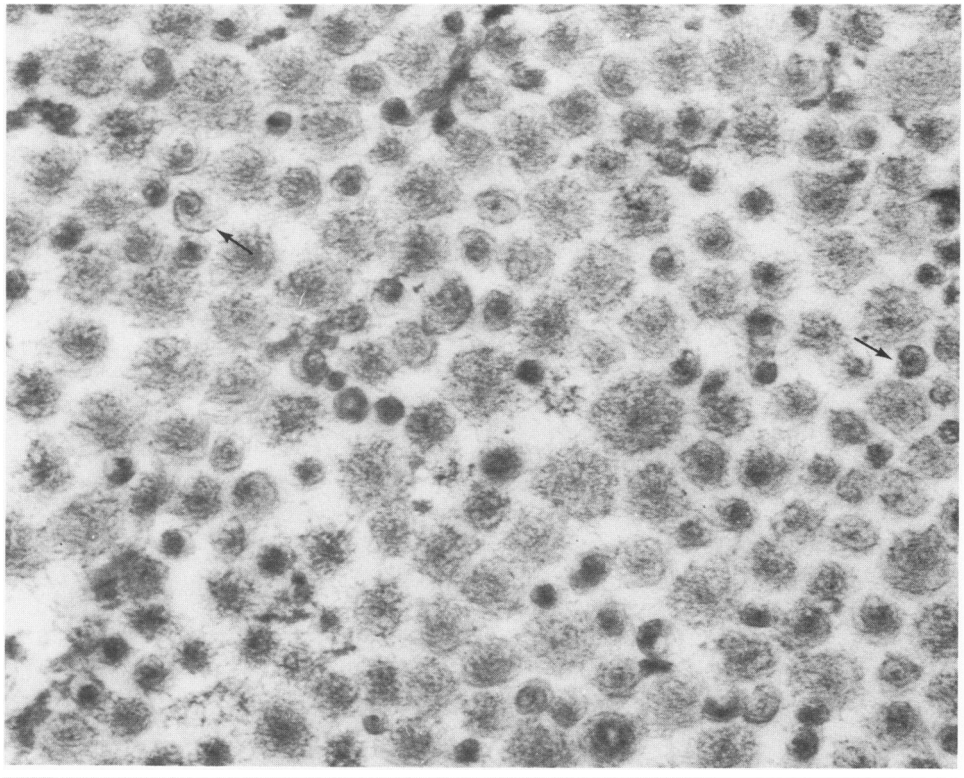
Figure 8—Cross-sections of collagen fibrils from incubation with urea, stained only with ruthenium red. Positive staining occurs only in raveled segments or those just beginning to unwind, seen as spiral configurations (*arrows*). (×50,000)

Figure 9—Long sections of urea-treated collagen. Note the absence of ruthenium red staining along fibrils. (×50,000)



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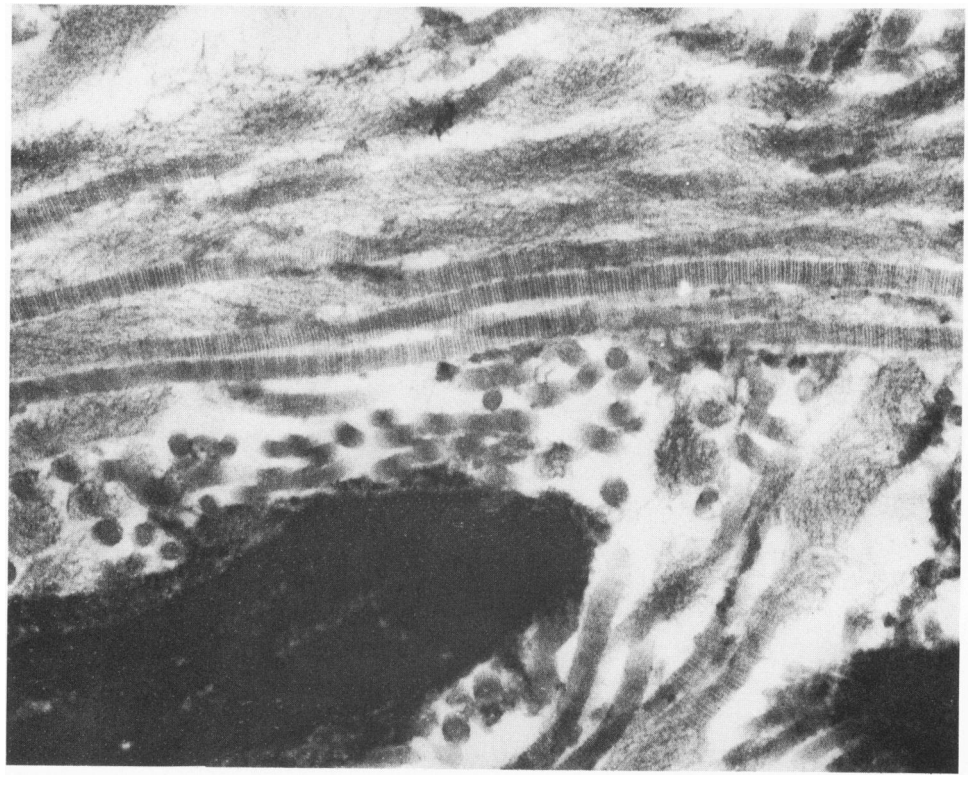

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