Some Properties of the Microfibrils of Vascular Elastic Membranes in Normal and Copper-Deficient Swine

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ABNORMALITIES IN THE STRUCTURE AND PROPERTIES of vascular elastin in copper deficiency are well established.[1-3] These studies have led to the hypothesis that there is a block in elastin biosynthesis at the step of intermolecular cross-linking of a soluble elastin precursor.[4] As a result there is an accumulation of salt-soluble proteins in the aorta,[5,6] one component of which has the amino acid composition predicted for a noncross-linked elastin precursor.[7] At the same time there is an accumulation of microfibrils on the surface of elastic membranes [8] and without detectable change in the structure of the preformed matrix.[8,9]

The present study of the solubility behavior toward strong salt solutions and elastase of the microfibrils of the vascular elastic membranes was undertaken to determine their relationship to the soluble protein and to the insoluble elastin.

Materials and Methods

Twelve pigs representing two litters of mixed breed animals were used in two separate experiments. In each experiment, the pigs were weaned at 5 days on an evaporated milk diet with mineral supplements.[10] The 6 control pigs received copper supplements after weaning. Selected deficient pigs were given copper sulfate supplements for 2 days preceding sacrifice, as previously described. The animals were weighed, and the volume of packed red cells (VPRC) was determined each week. Serum copper levels were assayed at least once each month by atomic absorption spectrophotometry on a Perkin-Elmer 303 instrument. At intervals determined by the severity of copper deficiency, animals were sacrificed by exsanguination under pentobarbital anesthesia.

Segments of right coronary artery were removed and cut into 0.5-mm. rings. After the treatments to be described, these were placed immediately into 3% glutaraldehyde with phosphate buffer pH 7.4, fixed overnight, postfixed with 1% osmium tetroxide, dehydrated, stained with 1% phosphotungstic acid in absolute alcohol (PTA) before infiltration, and embedded in Araldite. Ultrathin sections were stained with uranyl acetate before examining in a Siemens Elmiskop IA.

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Adjacent rings were fixed in Helly's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E) or Verhoeff's elastic-van Gieson (EVG) stains. Control rings were fixed and processed in the same way without other treatment.

In the first experiment, the arterial rings were placed in cold 1 M sodium chloride in phosphate buffer pH 7.4, for 1, 6, 12, or 24 hr., with agitation at 4°C before processing as described above. Adjacent rings were agitated at 4°C for 24 hr. in distilled water and similarly processed. In the second experiment arterial rings were incubated at 37°C without agitation for 15, 30, 60, or 120 min. in 2 ml. of tricine buffer (pH 7.8)[11] containing 0.2 mg. of purified Worthington elastase. The advantage of tricine over more alkaline buffers in preserving tissue architecture was important for ultrastructural studies. The elastase activity was monitored by simultaneous orcein-elastin incubations.[12] All elastase incubations were halted by the addition of a 0.5 M phosphate buffer solution (pH 5.9) to the incubation mixture. Identical 120-min. incubations without elastase were performed for controls. All arterial specimens from the second experiment were washed in buffer and processed for light and electron microscopy as described above.

Results

The vital data at termination of the experiments are shown in Table 1. Six control animals aged 77–114 days had normal serum copper levels and VPRC. Four deficient animals had extremely low serum copper levels and moderate reduction in VPRC. Two animals deficient up to the ages of 86 and 105 days, which received copper supplements for 2 days, had substantial increases in serum copper over the deficient pigs, but the VPRC remained in the same range as that of the deficient animals. The principal differences in the structure of the vessels of these

Table 1. Vital Data at the End of the Experiment

Pig No.	Age (days)	Body weight (kg.)	VPRC	Serum copper (μg./100 ml.)
		Cu Supplemented from Weaning		
136	98	27.6	40	207.7
137	77	14.6	37.5	242.2
139	114	29.6	43.5	250.1
152	100	20.5	36.0	195.0
153	87	14.5	35.0	211.0
154	81	12.6	37.0	190.0
		Cu Deficient		
131	105	26.4	26.0	8.8
156	79	12.2	17.0	4.9
158	98	24.0	22.5	6.0
160	106	22.8	21.5	8.8
		Cu Deficient, Supplemented 2 days		
135	107	30.0	22.0	47.0
155	88	14.8	18.5	52.0

three groups were like those described previously. Copper deficiency produced defects in the internal elastic membranes.[13] These were accompanied by an accumulation of PTA-staining microfibrils on the surfaces of the narrowed elastic membranes.[8] Copper supplementation appeared to produce a rapid increase in the quantity of the microfibrils. The reactions of the microfibrils to the treatments described were apparently the same regardless of whether they were obtained from control or copper-deficient pigs.

The light microscopic observations of control arteries are depicted in Fig. 1–4. In the control segments, fixed immediately and stained with EVG, the elastic fibers were conspicuous black bands (Fig. 1). After treatment with buffer for 2 hr., there was no discernible effect upon the elastic tissue (Fig. 2). In most instances, 1 hr. of elastase treatment was sufficient to dissolve all arterial elastic fibers, and digestion was always complete after 2 hr. (Fig. 3). Arterial segments after prolonged agitation in cold molar sodium chloride or in distilled water revealed no changes in the morphology and staining of the elastic fibers (Fig. 4). These treatments did produce medial condensation or swelling related to osmolarity of the medium.

Similar observations were made at the ultrastructural level. Arterial segments placed immediately into fixative provided the standard of reference. The internal elastic membrane (IEM) appeared as a thick band beneath the endothelium (Fig. 5). The subendothelial space, containing amorphous material and occasional collagen fibrils, was expanded in the deficient animals, as reported earlier.[8] Collagen was more conspicuous in the superficial media where large fibers were interwoven with smooth-muscle cell processes. At high magnification, a mantle of microfibrils was seen on the luminal surface of the IEM (Fig. 6). Most of the elastic membrane was poorly stained and was without discernible structure. This structureless material constitutes the homogeneous matrix of elastic fibers.

The ultrastructure of the elastase-treated segments is shown in Fig. 7–9. The elastic fibers were completely stainable with PTA after 15 min. of elastase digestion (Fig. 7). The fibers were irregular, and the digestion produced a longitudinal network of canals around those microfibrils incorporated into the elastic fibers. Microfibrils on the surface of elastic fibers were intact, and collagen fibrils were unaltered. After 30 min. of elastase treatment, the homogeneous matrix of elastic fibers was largely dissolved by the elastase, while microfibrils associated with the remnants of fiber matrix were intact (Fig. 8). One hour of elastase digestion removed all traces of the homogeneous matrix from the elastic fibers,

which were identified by residual bands of microfibrils (Fig. 9). Collagen fibrils appeared normal, but the structure of smooth-muscle cells was distorted by the prolonged incubation.

The saline extraction and distilled-water procedures caused no obvious alteration in the ultrastructure of the elastic tissue (Fig. 10). Both components were normal, although the microfibrils were particularly distinct. The extracellular, interfibrillar matrix of the saline-extracted arterial segments appeared to have been removed (compare Fig. 10 and 6).

Discussion

The normal process of elastogenesis, described in developing fetal arteries [14] and tendons [15] and in bovine ligamentum nuchae,[16] is characterized by the appearance of bundles of parallel microfibrils in the intercellular substance followed by the focal condensation among them of a homogeneous electron-lucent material that coalesces into the mature elastin matrix. The nature of these microfibils and their role in the process of elastogenesis are matters of much interest.

Ross and Sandberg [17] have studied the susceptibility to proteolytic enzymes of the microfibrils of elastic fibers obtained from fetal calf ligamentum nuchae by extraction with guanidine-HCl and purified collagenase.[17] The microfibrils were completely digested by a trypsin-chymotrypsin mixture or by elastase, and were partially digested by pepsin, while the amorphous elastin matrix was digested only by elastase. They concluded that the fibrils may be a fibrous protein distinct from elastin.

The present experiments indicate that the two ultrastructural components of elastic fibers in copper-deficient arteries are also distinct chemically, as well as morphologically. The native homogeneous, electron-lucent matrix, which composes most of the fiber, is very sensitive to elastase digestion, being attacked rapidly by the enzyme. When adequately exposed, this matrix is completely digested within 1 hr. The microfibrils on the surface of the elastic fibers and those embedded within elastic fibers, by comparison, are resistant to elastase digestion. They can be identified even after 2 hr. of exposure to the enzyme when the fiber matrix has been completely dissolved. The results of elastase digestion suggest, therefore, that the homogeneous matrix of elastic fibers corresponds to the unique protein elastin, while the microfibrils are not elastin. Similar results have already been reported using normal pig coronary arteries.[18] Moreover, the solubility experiments indicate that the excessive microfibrils of the copper-deficient arterial elastin [8] do not represent the presumed elastin precursor that may be extracted from

these arteries with molar sodium chloride.[7] The microfibrils are insoluble in molar sodium chloride and in water.

Earlier work of other investigators has demonstrated an apparently fine fibrillar internal component of elastic fibers about 200–250 Å wide that was relatively resistant to elastase.[19,20] While these may have been the same microfibrils studied by later investigators, it is impossible to be certain of their identity because of differences in the manner of preparation and examination. The earlier preparations were made from mature aorta and ligamentum nuchae by rigorous methods of purification and were examined in shadow-cast electron micrographs. At least they were not to be considered native chemical substances, and their thickness was doubtless exaggerated.

The present experiments demonstrate the practicability of separating the microfibrils of elastic tissue from the homogeneous elastin matrix by the action of elastase, and they offer the basis of a suitable isolation procedure. Attention is called to the merit of tricine buffer at pH 7.8 as a medium for elastase digestion, permitting the satisfactory preservation of fine structure for electron microscopic control of enzymic action.

Summary

Some properties of the microfibrils of the elastic membranes of copper-deficient pig coronary artery have been compared with those of normal and copper-supplemented animals. The microfibrils are insoluble in water and in cold molar sodium chloride, which extracts a soluble elastin precursor. They are comparatively resistant to elastase, which completely solubilizes the homogeneous elastin matrix. It is concluded that the microfibrils are chemically, as well as structurally, distinct from both the soluble elastin precursor and the insoluble elastin. These properties of the microfibrils of the copper-deficient animals were indistinguishable from those of the controls.

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[*Illustrations follow*]

Legends for Figures

Fig. 1–4 are photomicrographs of coronary artery from a control pig. The material was stained with Verhoeff's elastic and van Gieson stains and are at a magnification of × 225.

Fig. 5–10 are electron micrographs of coronary arteries of copper-deficient pigs. The arteries were fixed in glutaraldehyde and stained with phosphotungstic acid (PTA) and uranyl acetate.

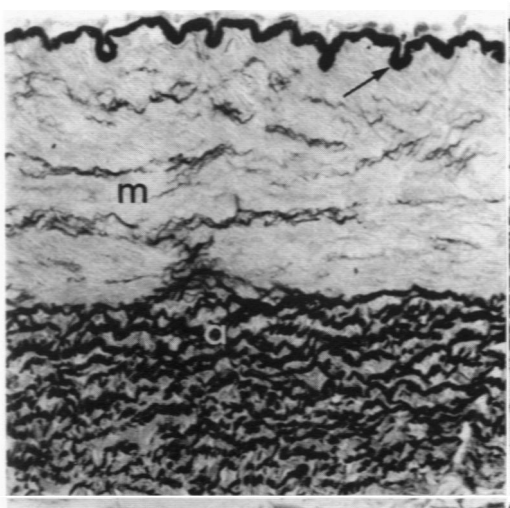
Fig. 1. Artery fixed immediately without other treatment. Internal elastic membrane *(arrow)* and elastic fibers of media *(m)* and adventitia *(a)* are stained black.

Fig. 2. Artery incubated for 2 hr. in buffer without elastase. Elastic tissue appears unaffected.

Fig. 3. Artery incubated in elastase for 2 hr. All elastic tissue has been digested.

Fig. 4. Artery extracted in cold 1 M NaCl for 24 hr. with no apparent effect on stainable elastic tissue.

Fig. 5. Artery fixed without prior treatment shows alterations characteristic of copper deficiency. There is a thick mantle of PTA-stained microfibrils on intimal surface of internal elastic membrane *(i)*. Collagen fibers *(c)* and smooth-muscle cells *(s)* are shown external to *(i)*. × 8000.



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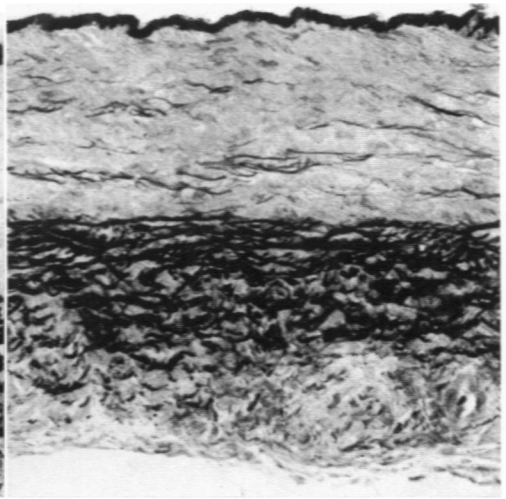

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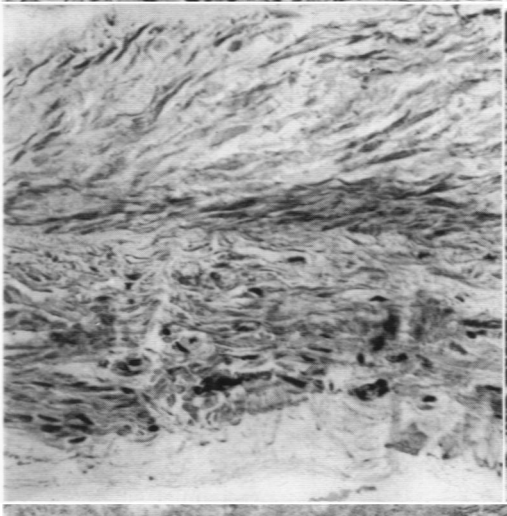

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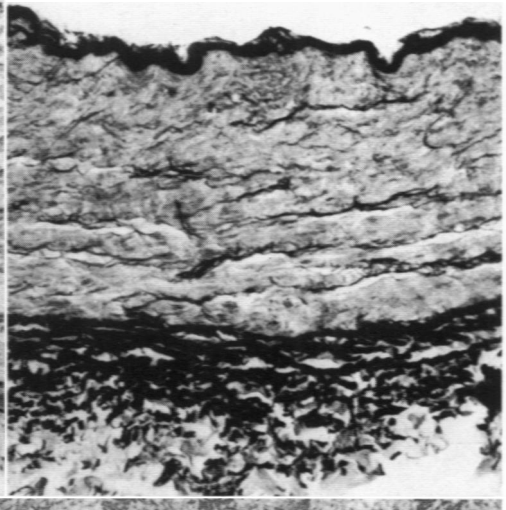

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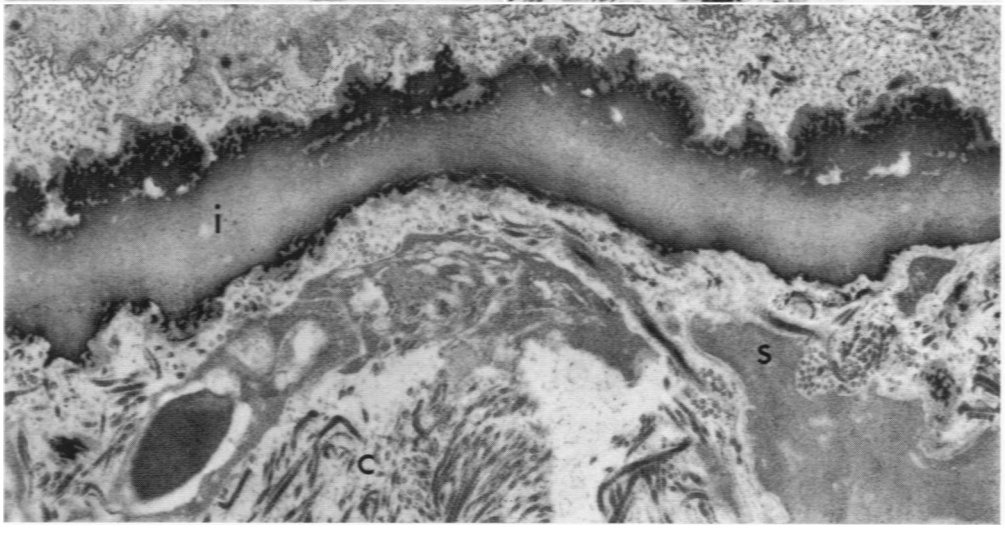

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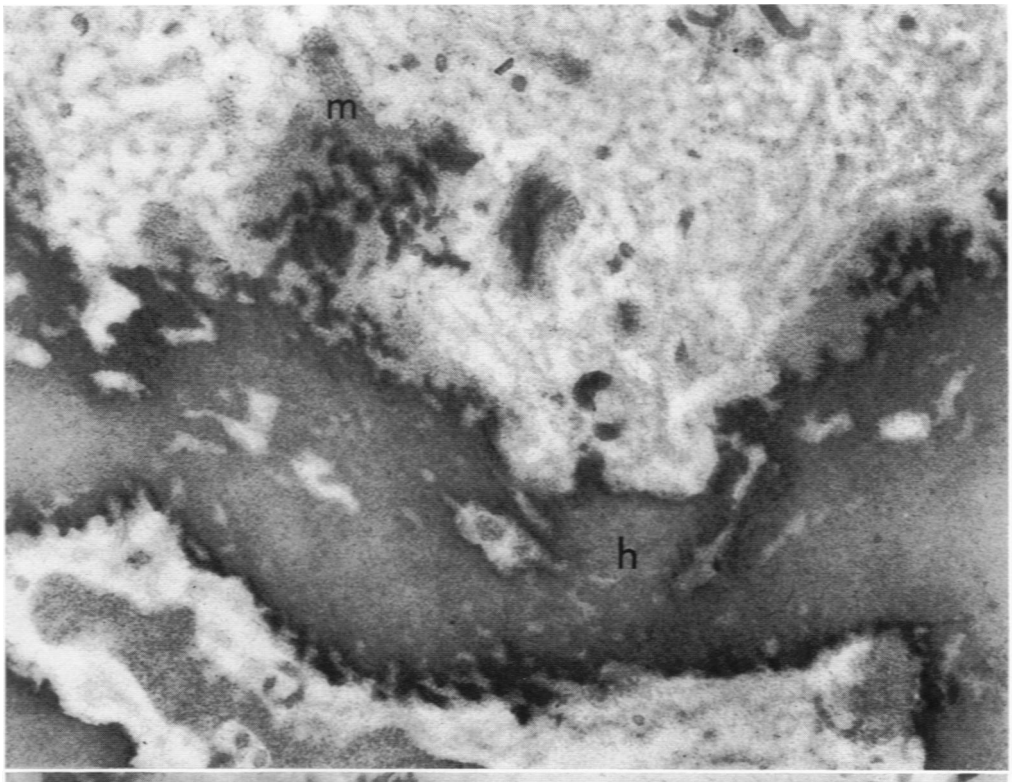
Fig. 6. At higher magnification, microfibrils *(m)* are distinguished from homogeneous matrix *(h)* of internal elastic membrane of an untreated artery. × 40,000.

Fig. 7. After 15-min. treatment with elastase, homogeneous matrix of elastic membrane stains more deeply with PTA and shows coarse canalization about the microfibrils *(arrows)* incorporated within it. Microfibrils *(m)* on surface of elastic membrane and collagen fibers *(c)* are intact but spread apart by an expanded interstitial space. × 40,000.



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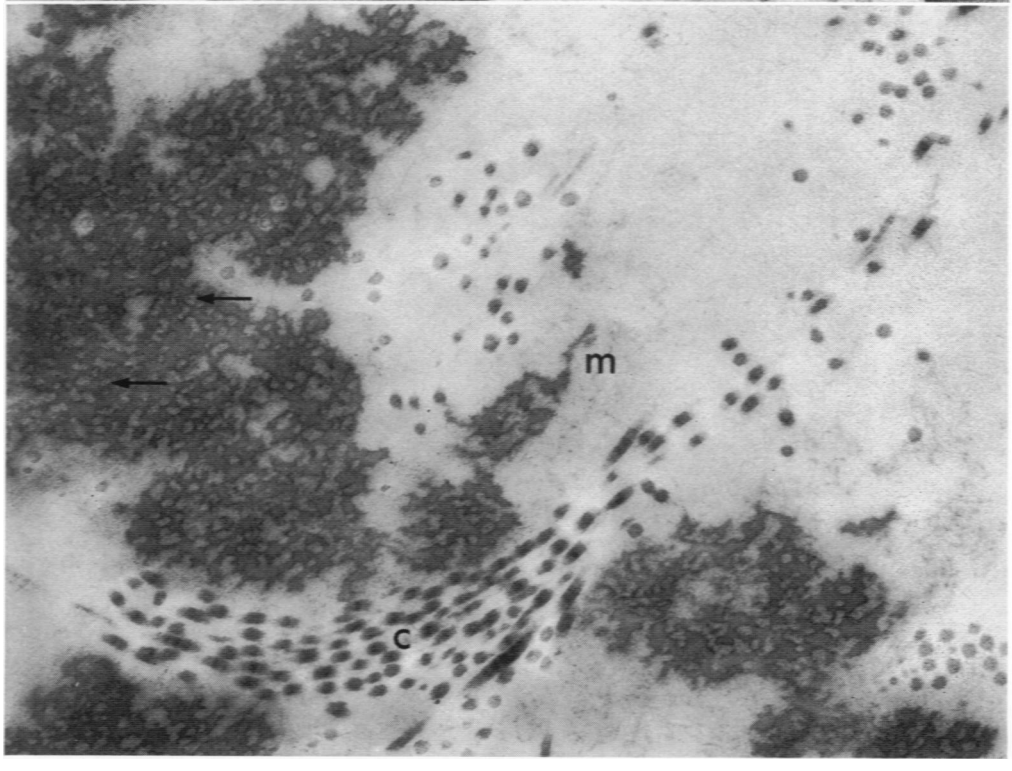

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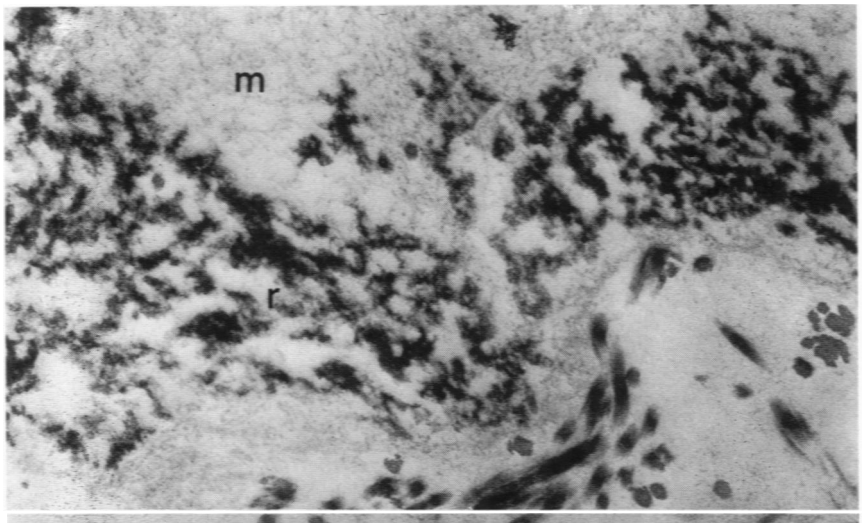
Fig. 8. After 30-min. treatment with elastase, homogeneous matrix of elastic membrane has largely dissolved, leaving deeply staining reticular residue *(r)*, network of microfibrils *(m)*, and intact collagen fibers. × 40,000.

Fig. 9. After 1 hr. of elastase treatment, no homogeneous elastic matrix remains, but a network of microfibrils marks its former site. Collagen fibers are unaltered, and a process of smooth-muscle cell *(s)* may be distinguished. × 40,000.

Fig. 10. After 24-hr. extraction in cold, buffered molar sodium chloride, homogeneous matrix and microfibrils of internal elastic membrane are apparently unaltered, but microfibrils are shown in bolder relief by the swelling and clearing of the interstitial space. × 40,000.



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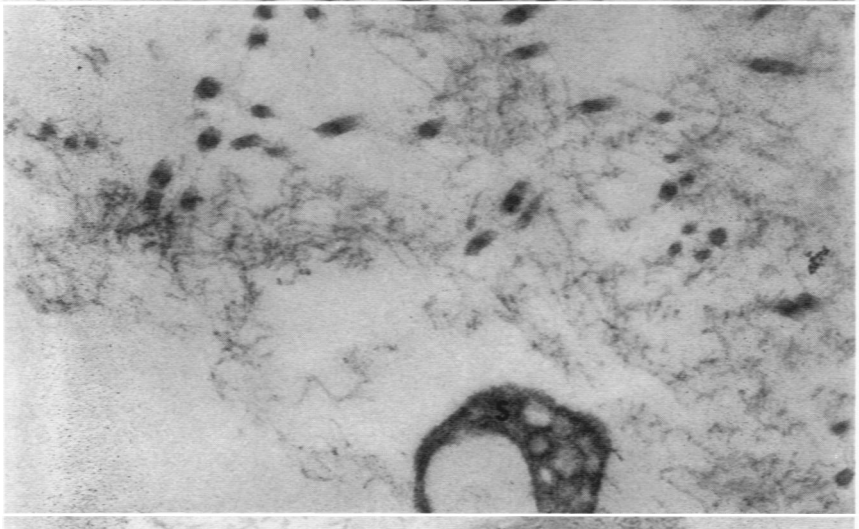

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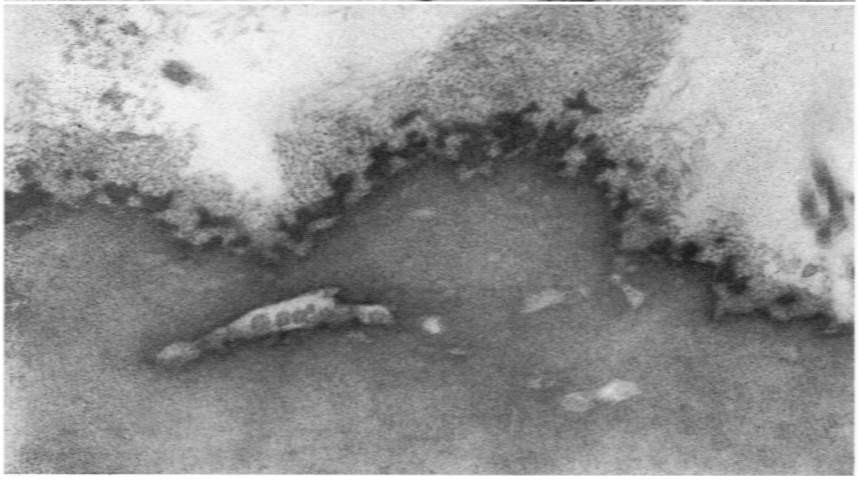

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