THE FINE STRUCTURE OF ELASTIC FIBERS

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ABSTRACT

The fine structure of developing elastic fibers in bovine ligamentum nuchae and rat flexor digital tendon was examined. Elastic fibers were found to contain two distinct morphologic components in sections stained with uranyl acetate and lead. These components are 100 A fibrils and a central, almost amorphous nonstaining area. During development, the first identifiable elastic fibers are composed of aggregates of fine fibrils approximately 100 A in diameter. With advancing age, somewhat amorphous regions appear surrounded by these fibrils. These regions increase in prominence until in mature elastic fibers they are the predominant structure surrounded by a mantle of 100 A fibrils. Specific staining characteristics for each of the two components of the elastic fiber as well as for the collagen fibrils in these tissues can be demonstrated after staining with lead, uranyl acetate, or phosphotungstic acid. The 100 A fibrils stain with both uranyl acetate and lead, whereas the central regions of the elastic fibers stain only with phosphotungstic acid. Collagen fibrils stain with uranyl acetate or phosphotungstic acid, but not with lead. These staining reactions imply either a chemical or an organizational difference in these structures. The significance and possible nature of the two morphologic components of the elastic fiber remain to be elucidated.

INTRODUCTION

The fine structure of large mature elastic fibers[1] has been most frequently described from intact specimens of artery, lung, and ligamentum nuchae, and from material chemically isolated from these structures. It is the purpose of this paper to describe the fine structure and staining characteristics of in vivo, early, immature elastic fibers and to follow their subsequent development and maturation to the larger more commonly observed elastic fibers seen in various connective tissues. These observations represent the fine structure of developing elastic fibers in fetal bovine ligamentum nuchae and fetal and postnatal rat digital flexor tendon.

Authors of previous studies have generally agreed that elastic fibers demonstrate no cross-banding or clear fibrillar substructure in the electron microscope. Partridge (20), Gotte and Serafini-Fracassini (5), Hall et al. (6, 7), and Lansing

[1] The term *elastic fiber* as used in this paper refers to a morphologic entity that corresponds to fibers seen in the light microscope after staining with the various elastic stains (aldehyde fuchsin, or orcein, etc.). The exact chemical composition of *elastic fibers* is not yet known. In contrast, the term *elastin* is used to describe the protein residue which is found after the various chemical extractions of connective tissue used to isolate this protein, and which demonstrates a somewhat constant amino acid composition. The term *elastin* will be used therefore, to indicate material that has been prepared by one of these chemical methods or to indicate the postulated characteristic protein of elastic tissues.

et al. (13) observed a fibrillar structure in isolated elastin[1] that was in the range of 100 to 200 A in diameter, whereas Cox and Little (3) denied seeing any fibrils. A fibrillar component associated with either the surface or the interior of the elastic fiber has been described in intact vessels (8, 9, 11, 12, 18, 19), in the lung (10, 14, 22), in the skin (1), in the ligamentum nuchae (23), and in other connective tissues (14, 16). In contrast, Cox and Little (3) observed no fibrillar substructure in any of these tissues.

Recognition of immature elastic fibers in intact tissues has been complicated by the lack of specific morphologic criteria. Observations of chemically isolated elastin are also difficult to interpret, owing to potential artifacts or loss of structure arising from the relatively extreme methods of isolation. This report presents specific morphologic criteria for the identification of both mature and immature elastic fibers in tissue sections prepared for electron microscopy.

MATERIALS AND METHODS

Two different connective tissues containing elastic fibers were examined with the electron microscope. These consisted of (*a*) ligamentum nuchae from fetal calves ranging in age from one to nine months; (*b*) flexor digital tendons from 15- and 18-day-old fetal, and from newborn, 5- and 30-day-old Sprague-Dawley rats. Several modes of tissue preparation were employed and compared. These included:

1. Fixation in osmium tetroxide buffered with s-collidine (2) (pH 7.3).
2. Fixation in osmium tetroxide buffered with s-collidine (pH 7.3), and postfixation with neutral buffered formalin.
3. Fixation in 6% glutaraldehyde with cacodylate

FIGURE 1 This micrograph represents an area from part of a 4 month fetal ligamentum nuchae. The elastic fibers and collagen fibrils (*co*) are obliquely sectioned. The elastic fibers consist of fibrils (*f*) (approximately 100 A) that surround central nonstaining areas (*ca*). Fixed with OsO_4 followed by formalin. Stained with uranyl acetate and lead. × 29,000.

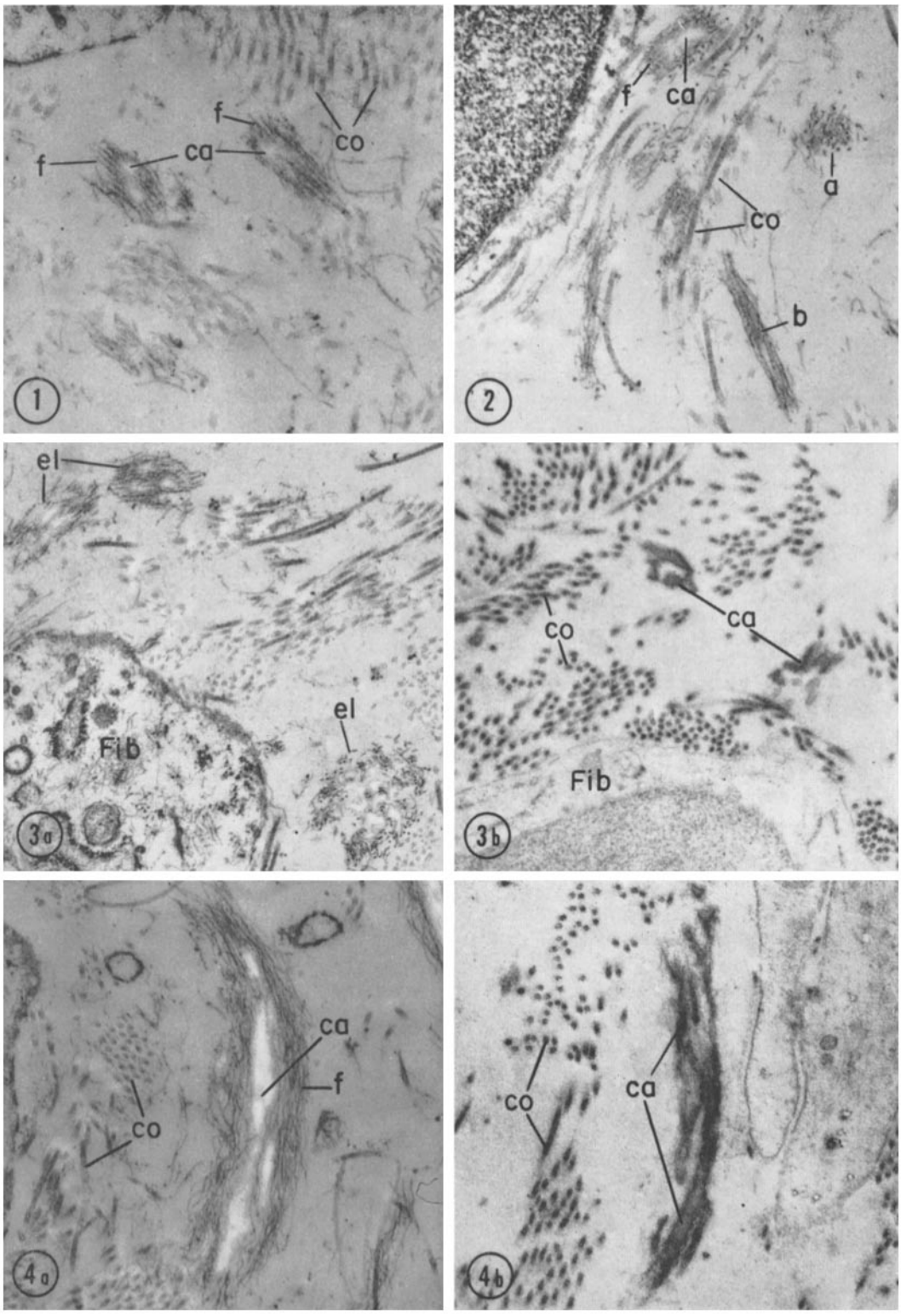
FIGURE 2 Another example of a 4 month fetal ligamentum nuchae containing several elastic fibers. A cross-section (*a*) and a longitudinal section (*b*) of elastic fibers consisting of only 100 A fibrils, and a larger fiber cut obliquely with prominent central, nonstaining areas (*ca*) surrounded by dark staining fibrils (*f*) can be seen. Collagen fibrils (*co*) approximately 330 A in diameter are also demonstrated. Fixed with OsO_4 followed by formalin. Stained with uranyl acetate and lead. × 29,000.

FIGURE 3 *a* A micrograph from the same specimen as shown in Figs. 1 and 2. Note the elastic fibers (*el*) with their central nonstaining areas surrounded by dark staining fibrils; compare with Fig. 3 *b*. Part of a fibroblast (*Fib*) is seen in one corner of the micrograph. Fixed with OsO_4 followed by formalin. Stained with uranyl acetate and lead. × 20,500.

FIGURE 3 *b* This is a section adjacent to that in Fig. 3 *a* stained with phosphotungstic acid. Phosphotungstic acid stains the central areas (*ca*) of the elastic fiber intensely, whereas the mantle of fibrils can hardly be identified. The collagen fibrils (*co*) are also densely stained with this technique. Part of a fibroblast (*Fib*) with a portion of its nucleus is also visible. Fixed with OsO_4 followed by formalin. Stained with 1% aqueous phosphotungstic acid. × 20,500.

FIGURE 4 *a* This longitudinal section of an elastic fiber from a 6 month fetal ligamentum nuchae shows an increase in the ratio of the central nonstaining area (*ca*) to the peripheral fibrils (*f*). The collagen fibrils (*co*) are similar in diameter to those in the younger animals seen in the preceding micrographs. Fixed with OsO_4 followed by formalin. Stained with uranyl acetate and lead. × 21,000.

FIGURE 4 *b* A section adjacent to that seen in Fig. 4 *a* stained with phosphotungstic acid. This demonstrates the dense staining of the central areas (*ca*) of the elastic fiber and the absence of stain in the peripheral fibrils. Collagen fibrils (*co*) are densely stained with PTA. Fixed with OsO_4 followed by formalin. Stained with 1% aqueous phosphotungstic acid. × 20,000.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buffer (pH 7.3), and postfixation in osmium tetroxide buffered with s-collidine (pH 7.3).

All tissues were embedded in epoxy resin (15). Thin sections were examined after staining at room temperature with (*a*) uranyl acetate (3% aqueous solution) for 2 hr; (*b*) Millonig's lead for 10 min (17); (*c*) uranyl acetate and lead; and (*d*) 1% aqueous phosphotungstic acid (PTA) for 1 hr. Unstained sections were examined and compared with the various stained materials in an RCA EMU 3G electron microscope.

Paraffin sections and 1 μ epoxy sections were stained with Gomori's (4) aldehyde fuchsin for light microscopy.

OBSERVATIONS

The development of the cells and their interrelationships with the extracellular fibers in each of these systems will be presented in subsequent reports. This study will describe and characterize the fine structure appearance of the elastic fibers in these two systems.

Calf Ligamentum Nuchae

Fine Structure of Fibers

Cross-sections of developing 4-month-old fetal calf ligamentum nuchae demonstrate two populations of fibrils. These consist of typical collagen fibrils, approximately 300 to 350 A in diameter, and fine fibrils, approximately 100 A in diameter, that are grouped in bundles of eight to twenty fibrils, usually surrounding a less dense central zone, when stained with uranyl acetate and/or lead (Figs. 1, 2, and 3 *a*).

At 4 to 6 months, the elastic fibers demonstrate the cross-sectional appearance described above (Figs. 1 and 3 *a*). In longitudinal sections the central less dense areas are only visible if the fibers are cut on a bias (Figs. 1, 2, 3 *a*, and 4 *a*). Otherwise, the longitudinal sections demonstrate bundles of fibrils lying in parallel array (Fig. 2).

Between 6 and 9 months, a marked change occurs in the appearance of the elastic fibers (Figs. 5 *a*, 5 *b*, and 6). This consists of an increase in size in the central less dense areas that are always surrounded by a mantle of fibrils each approximately 100 A in diameter. In some regions these fibrils appear to be beaded in a regular fashion (Figs. 5 *a* and *b*), and in cross-section they have a tubular profile (Fig. 6).

Staining Characteristics of Elastic Fibers

When sections of ligamentum nuchae are stained with phosphotungstic acid, the peripheral mantle of fibrils of the elastic fibers is difficult to

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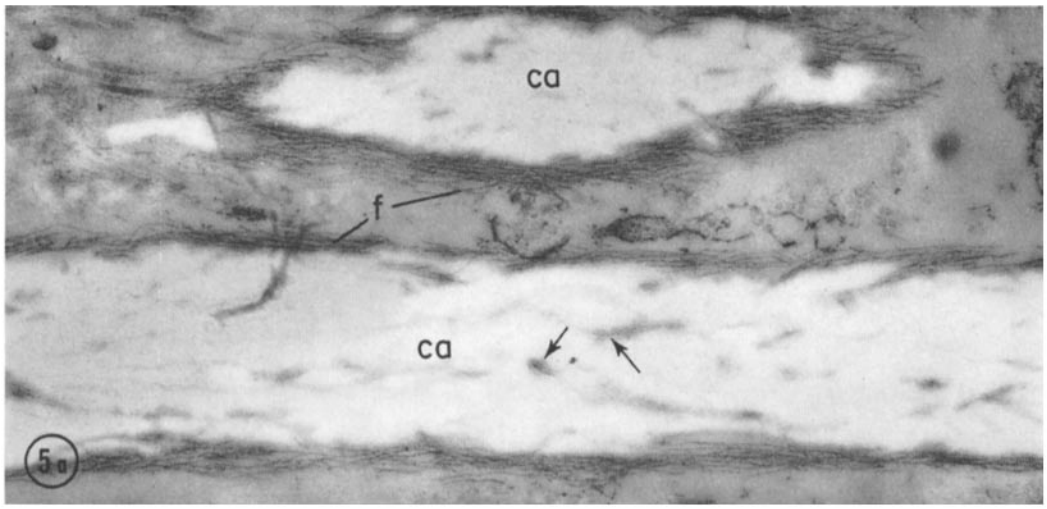
Figure 5 *a* The central nonstaining areas (*ca*) have increased markedly in size in this 8-month fetal ligamentum nuchae compared to those seen in Fig. 4 *a*. The 100 A fibrils (*f*) are present as a mantle around the fiber and can be seen within the interstices of the central area (*ca*) as well (arrows). In some regions the fibrils appear to have a beaded appearance. Fixed with OsO_4 followed by formalin. Stained with uranyl acetate and lead. × 34,500.

Figure 5 *b* The central area (*ca*) of the elastic fiber from an 8-month fetus demonstrates a greater affinity for the stain when the ligamentum nuchae is fixed with glutaraldehyde as compared with primary OsO_4 fixation seen in Fig. 5 *a*. Fixed with glutaraldehyde followed by osmium tetroxide. Stained with uranyl acetate and lead. × 34,500.

Figure 6 This cross-section of an 8-month fetal ligamentum nuchae demonstrates two elastic fibers of different size. The larger fiber has a central nonstaining area (*ca*), which is continuous and irregular in shape, and both peripheral and centrally located 100 A fibrils (*f*). The fibrils in the deep recess of the fiber account for the appearance of centrally placed fibrils in longitudinal sections (Figs. 5 *a* and *b*). The smaller elastic fiber on the right contains three separate central areas (*ca*) each of which is surrounded by fibrils (*f*). This fiber is similar to the elastic fibers in the rat tendon seen in Figs. 8 *a* and 9 *a*. Fixed with OsO_4 followed by formalin. Stained with uranyl acetate and lead. The inset contains a higher magnification of several of the fibrils that surround and are embedded in the elastic fiber. These fibrils present a tubular profile and are similar to those in the rat tendon in Fig. 12. Fig. 6, × 56,500; Inset, × 153,000.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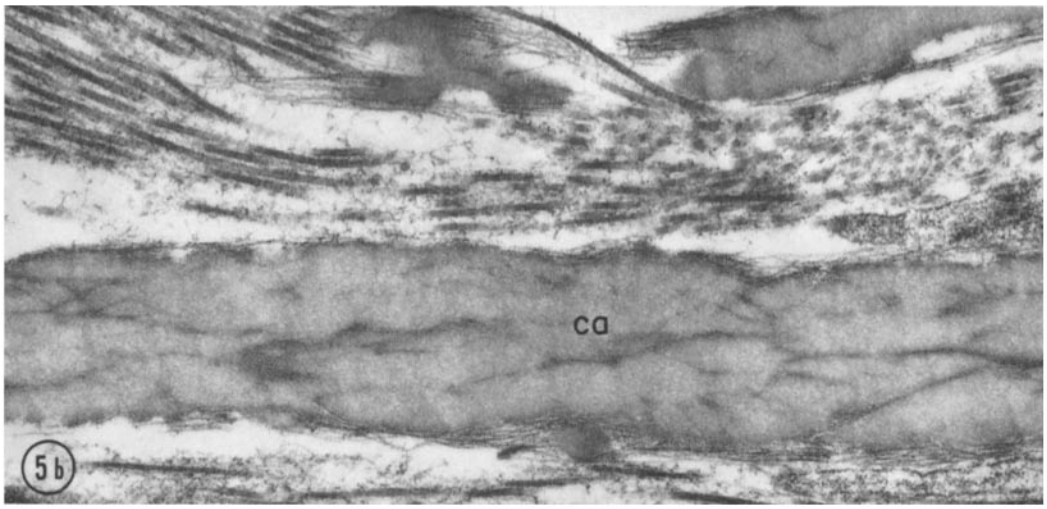
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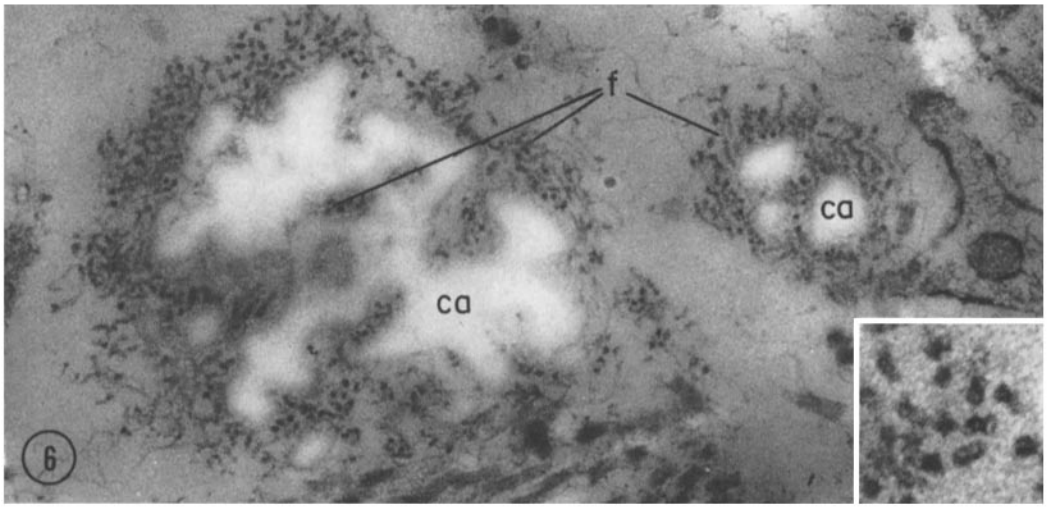
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see, as the fibrils do not stain. In contrast, the central areas that demonstrated no affinity for uranyl acetate and/or lead showed marked affinity for phosphotungstic acid, which stains them a dark gray color, slightly less intense than the staining of the collagen fibrils within the same sections (Figs. 3 *b* and 4 *b*). When these structures are fixed with glutaraldehyde and stained with lead and/or uranyl acetate, the central area appears somewhat denser than that seen in OsO_4-fixed tissues (Fig. 5 *b*).

Aldehyde fuchsin–positive fibers comparable in size and distribution to the elastic fibers in the electron micrographs were seen in light microscope sections.

Developing Rat Flexor Digital Tendon

Fine Structure Comparisons Between Tendon and Ligamentum Nuchae

Aldehyde fuchsin–positive fibers can be demonstrated in 1 μ epoxy sections of the flexor digital tendon of the newborn rat. These structures correspond to fine collections of fibrils in the electron micrographs (Figs. 8 *a*, 11, and 12) that are identical in appearance, in both cross- and longitudinal sections, to those seen and identified as developing elastic fibers in bovine ligamentum nuchae.

Cross-sections of these elastic fibers stained with lead and/or uranyl acetate consist of collections of fibrils, approximately 100 A in diameter, that

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Figure 7 A transverse section of an 18 day fetal rat digital flexor tendon. This region contains two collections of stained 100 A fibrils (*f*) that represent early or immature elastic fibers. Transversely sectioned collagen fibrils fill the remainder of the extracellular space. Several fibroblasts (*Fib*) are present. Fixed with OsO_4. Stained with uranyl acetate and lead. × 46,500.

Figure 8 *a* In this cross-section of a newborn rat tendon, the elastic fiber presents a picture similar to that seen in ligamentum nuchae in Figs. 1 to 3 *a*. Here, the 100 A fibrils (*f*) surround central nonstaining areas (*ca*). A common finding in tendon is the presence of the elastic fiber within a cellular niche. The plasma membrane (*pm*) and rough endoplasmic reticulum (*er*) of the fibroblast can also be seen. Fixed with OsO_4. Stained with uranyl acetate and lead. × 60,000.

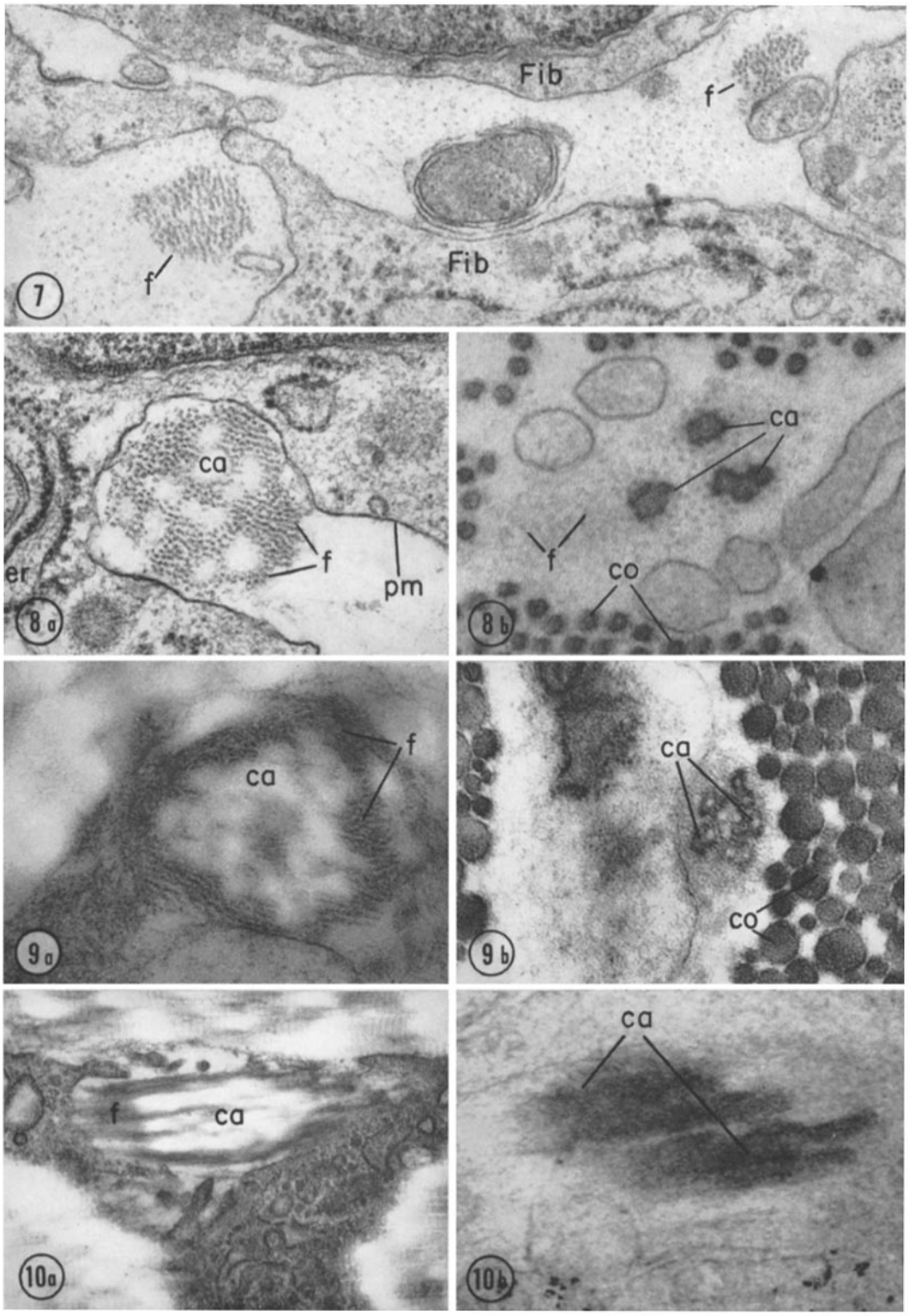
Figure 8 *b* A section adjacent to that in Fig. 8 *a* stained with phosphotungstic acid. The affinity of the central areas (*ca*) for this stain is striking, with the periphery being more heavily stained than the center. As in ligamentum nuchae (Fig. 3 *b*), the 100 A fibrils (*f*) are poorly stained. The collagen (*co*) is densely stained and many cell processes are evident. Fixed with OsO_4. Stained with phosphotungstic acid. × 74,000.

Figure 9 *a* An increase in the amount of the nonstaining central material (*ca*) in the elastic fiber can be seen in this cross-section from a 30-day rat tendon. The peripheral fibrils (*f*) are unchanged in size (about 100 A) and are obliquely sectioned in this micrograph. Fixed with OsO_4. Stained with uranyl acetate and lead. × 47,500.

Figure 9 *b* A PTA-stained cross-section adjacent to that seen in Fig. 9 *a*. The central areas (*ca*) in the elastic fiber are densely stained and the fibrils are poorly stained. The collagen (*co*) stains intensely and demonstrates a marked increase in fibril diameter at this age; compare with Fig. 8 *b*. Fixed with OsO_4. Stained with 1% aqueous phosphotungstic acid. × 25,000.

Figure 10 *a* This oblique section of a 30 day rat tendon contains an elastic fiber adjacent to a cell. The fine fibrils (*f*) surround a nonstaining area (*ca*) similar to that in Figs. 1 and 4 *a*. Fixed with OsO_4. Stained with uranyl acetate and lead. × 16,500.

Figure 10 *b* A much smaller elastic fiber (*el*) from a 30 day rat tendon is seen in this phosphotungstic acid–stained section. It is cut obliquely like the one in Fig. 10 *a*. The central areas are densely stained and the surrounding fibrils are not evident. Fixed with OsO_4. Stained with 1% aqueous PTA. × 100,000.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surround central nonstaining areas (Figs. 8 *a*, 9 *a*, and 12). Higher resolution micrographs of cross-sections of the individual fibrils demonstrate that they are nonhomogeneous in structure. They appear to consist of a light central core about 40 A in diameter surrounded by a dense circumferential layer about 30 A in thickness and to have a somewhat tubular appearance (similar to those in the ligamentum nuchae) (Figs. 6 and 12).

Longitudinal sections of elastic fibers appear as parallel aggregates of fibrils with no apparent cross-banding (Fig. 11). As with the ligamentum nuchae, when sections of tendon are stained with phosphotungstic acid, the long fibrils (100 A) are difficult to see in either cross- or longitudinal sections (Figs. 8 *b*, 9 *b*, and 10 *b*). Instead, the previously nonstaining central areas stain densely with PTA in a nonuniform fashion, in which the periphery appears denser than the center (Figs. 8 *b* and 9 *b*). This nonuniformity of the central areas after PTA staining is less evident in longitudinal sections (Fig. 10 *b*), probably owing to inclusion of the entire central structure within the section and consequent overlap of these regions. In 15-day and 18-day fetuses, most elastic fibers consist of collections of fibrils (100 A) apparently without central nonstaining areas (Fig. 7). The central areas become a prominent feature of the elastic fibers with increasing age (Figs. 8 *a* and 9 *a*).

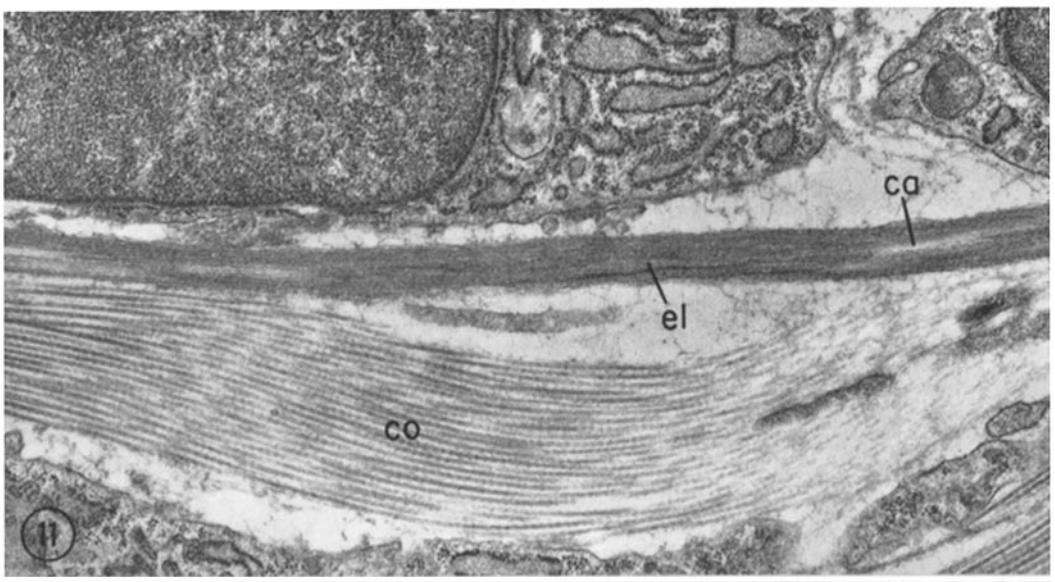
Comparative Staining of Elastic Fibers and Collagen

The staining characteristics of elastic and collagen fibers are most easily compared in transverse sections. The presence of large numbers of collagen fibrils lying parallel to elastic fibers in the digital tendon facilitates the study of the differential staining characteristics of these two connective tissue components.

Adjacent sections of OsO_4-fixed newborn rat flexor digital tendon were viewed in unstained sections and in sections stained with (*a*) 3% uranyl acetate, (*b*) Millonig's lead (17), (*c*) 1% aqueous phosphotungstic acid, and (*d*) both uranyl acetate and lead. In unstained sections, the collagen and the fibrillar portion of the elastic fiber were of similar density but, in contrast, the central area of the elastic fibers was less dense and similar in density to the background. In uranyl acetate–stained sections, both collagen and the 100 A elastic fibrils demonstrated similar staining, whereas the central areas of the elastic fibers appeared to be unstained (Fig. 13 *a*). A change in the relative densities of the collagen and the fibrillar portion of the elastic fiber was seen after lead staining. The fibrils of the elastic fibers were stained by the lead, whereas both the collagen and central areas of the elastic fibers were not stained and were even lower in density than the background material within the extracellular space (Fig. 13 *b*). Both the collagen fibrils and the central areas of the elastic fibers stained with phosphotungstic acid, whereas the 100 A fibrils of the elastic fiber were unstained with PTA as compared with the background (Fig. 13 *c*). After double staining with lead and uranyl acetate, the elastic fibers and collagen display a cumulative effect from the two stains (Fig. 13 *d*).

FIGURE 11 This micrograph shows a longitudinal section of a newborn rat digital flexor tendon in which a long elastic fiber (*el*) appears to consist primarily of densely stained 100 A fibrils with occasional lighter areas (*ca*) that represent the nonstaining central areas shown in Figs. 8 *a* and 12. A somewhat beaded appearance can be seen in the fibrils of the elastic fiber, similar to that seen in ligamentum nuchae. Collagen fibrils (*co*) with their characteristic banding can be seen. In this micrograph, the elastic fiber follows a straighter course than the collagen and lies in proximity to two fibroblasts. The straighter appearance of the 100 A fibrils in tendon as compared to ligamentum nuchae may be accounted for by the fact that all tendons were fixed in their extended positions while the ligaments were allowed to contract. Fixed with OsO_4. Stained with uranyl acetate and lead. × 17,500.

FIGURE 12 An elastic fiber from a newborn rat tendon viewed in cross-section at higher magnification. The 100 A fibrils (*f*) are not homogeneous, but appear as tubular profiles. The central nonstaining areas (*ca*) of the elastic fiber and fibroblast processes (*Fib*) are also seen. Fixed with OsO_4. Stained with uranyl acetate and lead. × 153,000.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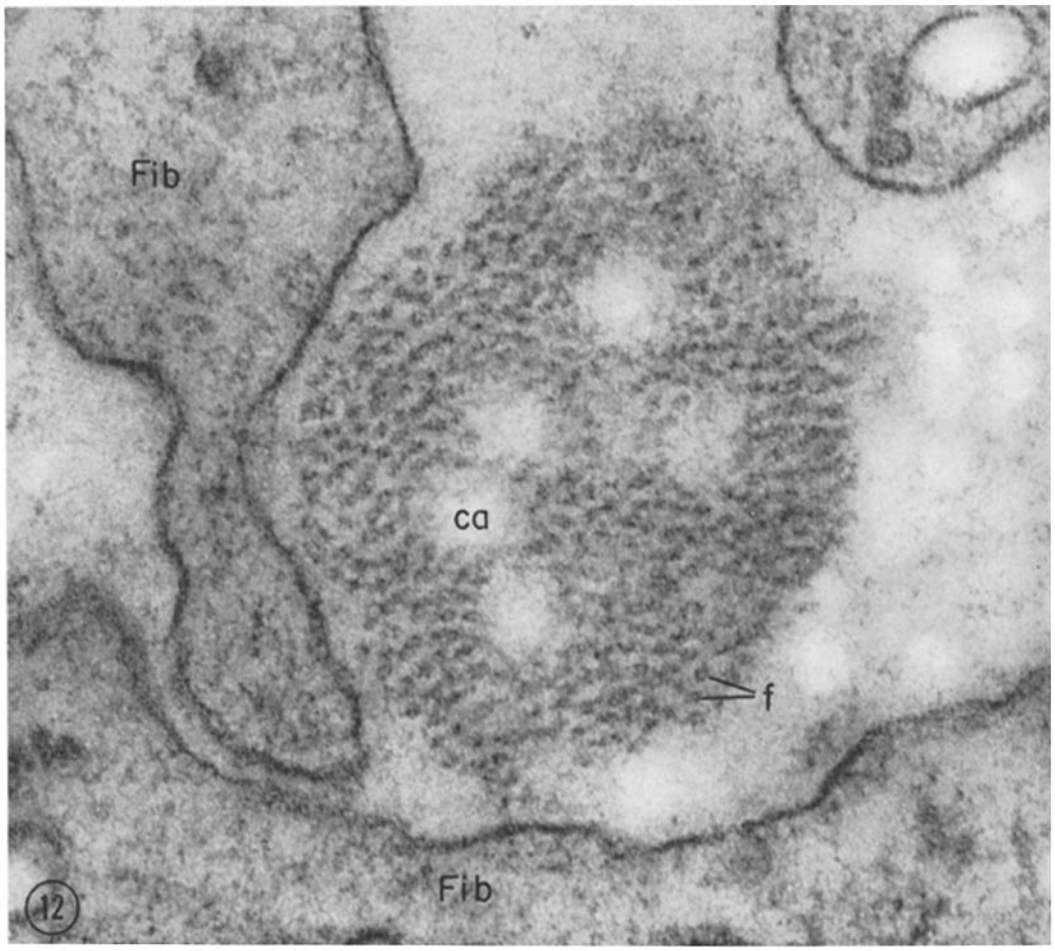
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The interpretation of these differential staining characteristics is difficult because information is lacking as to the specific chemical reactions associated with each of these stains. Some chemical or organizational difference between collagen, the fibrillar portion, and the central portion of the elastic fibers is implied by their different staining characteristics.

DISCUSSION

As a result of these investigations, it is possible to identify and characterize the fine structure of both young and mature elastic fibers. Mature elastic fibers contain two morphologic components: a large central region or core that does not stain with lead or uranyl acetate but does stain with phosphotungstic acid, surrounded by a thin layer of fine fibrils (each 100 A in diameter) that stain in opposite fashion to the central core. The recognition of mature elastic fibers and their development from collections of fine fibrils (100 A) within the ligamentum nuchae, a structure rich in elastic tissue, has enabled the identification of these collections of 100 A fibrils as young or immature elastic fibers. These observations have permitted the recognition of similar fibers in a tissue, such as the rat digital tendon, that contains relatively little elastin as determined chemically.

Studies of both ligamentum nuchae and tendon have demonstrated identical, fine structural characteristics in small or immature elastic fibers, in terms of both morphology and selective staining characteristics. Each immature elastic fiber consists of bundles of 100 A fibrils, that present tubular profiles when stained with uranyl acetate and/or lead. After early development these fibrils surround central regions that have no affinity for these two stains. In sections stained with phosphotungstic acid, the fibrils (100 A) do not stain, whereas the central regions demonstrate a marked affinity for PTA, and appear as nonuniform, dark gray zones. With increasing size, the central cores apparently coalesce, eventually comprise the majority of the fiber, and are surrounded by a thin layer of 100 A fibrils. Similar fine fibrils can be seen irregularly dispersed within the interstices of the central core.

Both components, the 100 A fibrils and the central area, are clearly integral parts of the elastic fiber, a finding which is in agreement with the findings of Haust and associates (8). In many studies of structures containing elastic fibers, phosphotungstic acid has been utilized to stain the tissues. In such situations, only the central, nonfibrillar portion of the fiber may be apparent. This would explain the failure of many investigators to recognize both the 100 A fibrils and the central areas as integral parts of the elastic fiber.

At present, there is no evidence as to the chemical nature of the two distinctly different morphologic components in elastic fibers. There is an

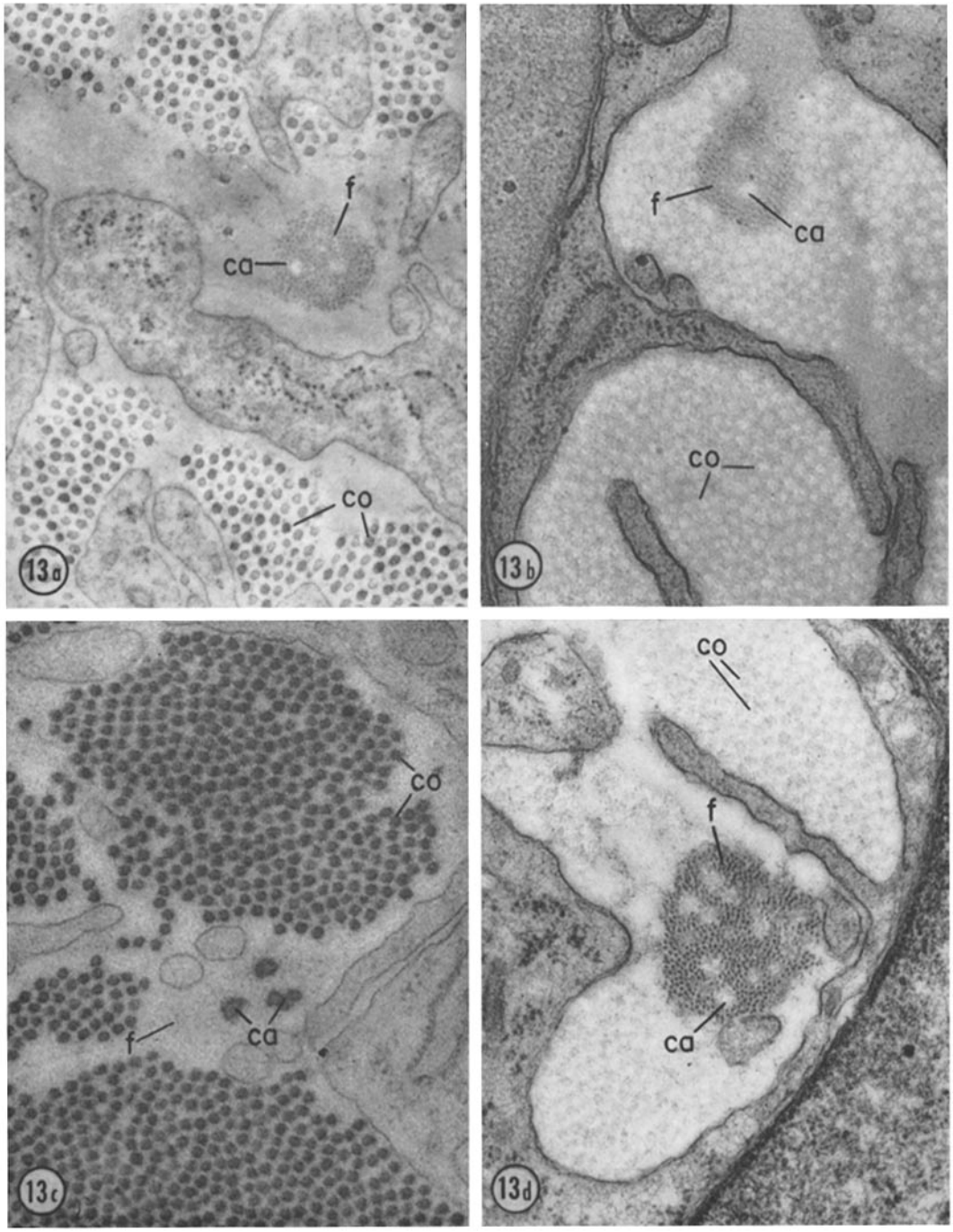
FIGURE 13 These micrographs represent adjacent cross-sections from a newborn rat tendon that have been stained differently in an attempt to characterize stain affinities of the collagen and the two components of the elastic fiber. All tissues were fixed with OsO_4.

FIGURE 13 *a* This section was stained with uranyl acetate. The 100 A fibrils (*f*) of the elastic fiber and the collagen fibrils (*co*) are of similar density, whereas the central areas of the elastic fiber (*ca*) are of the same magnitude of density as the background. × 37,000.

FIGURE 13 *b* After staining with lead alone, the fibrils (*f*) of the elastic fiber have a high density whereas the central areas (*ca*) of the elastic fiber and the collagen (*co*) are of similar density which is lower than that of the background. × 38,000.

FIGURE 13 *c* Phosphotungstic acid was the only stain used that increased the density of the central areas (*ca*) of the elastic fiber. Collagen (*co*) is also intensely stained with PTA. The fibrils (*f*) of the elastic fiber are poorly distinguished from the background. × 41,000.

FIGURE 13 *d* Double staining (uranyl acetate and lead) appears to be additive in that the fibrils (*f*) of the elastic fiber are more intensely stained than the central areas (*ca*) of the elastic fibers or the collagen (*co*). However, in this case the collagen has greater density than the background. × 41,000.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obvious difference in size and configuration between the 100 A fibrils and the central core. A possible chemical or organizational difference in their composition is implied by their differential staining: the fibrils stain with uranyl acetate and lead, and the central regions stain with phosphotungstic acid. At the pH's employed for both uranyl acetate staining and phosphotungstic acid staining, the former is cationic and the latter anionic. This difference in charge may be related to the selective staining characteristics demonstrated in this study.

Chemical data from studies of elastin indicate that it consists of a single protein (20), but the possibility of the loss of the fibrillar (100 A) component during the isolation procedures has not been ruled out. Micrographs in published studies of chemically isolated elastin do not show the fibrillar structures we have demonstrated. However, the handlings of these materials are so dissimilar that it is impossible to make any comparisons.

Some of the possible explanations for the appearance of the elastic fibers in this report include:

1. Both the fibrillar and central areas are "elastin" but each is in a different form, which would account for the distinctive morphologic and staining characteristics observed. Thus the central core of the mature elastic fiber would arise from the fine fibrils as a result of their transformation into the central core by a cross-linking mechanism similar to that postulated by Partridge (21).
2. Mature elastic fibers are composed of two different components. A fibrillar component is laid down first. In the central core these fibrils are later masked by a nonfibrillar substance that produces the amorphous appearance observed in this structure.
3. The fibrils are composed of a fibrous protein, polysaccharide, or macromolecular entity that may act as a nidus for the formation of "elastin," and is either removed or displaced as the central areas, presumably elastin, appear.

The resolution of these problems will require the separation and characterization of each of the two morphologic components in the elastic fiber.

Addendum

Since the submission and acceptance of this manuscript, a paper entitled The fine structure of the oxytalan fiber by G. G. Carmichael and H. M. Fullmer appeared in the *Journal of Cell Biology*, 1966, **28**, 33. The resemblance between the oxytalan fiber in Fig. 3, page 35, in their paper and the immature elastic fiber in Fig. 11 of the rat flexor tendon in our paper is striking.

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