

# The Hagfish Slime Gland Thread Cell

## I. A Unique Cellular System for the Study of Intermediate Filaments and Intermediate Filament-Microtubule Interactions

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**ABSTRACT** Thread cell differentiation in the slime gland of the Pacific hagfish *Eptatretus stouti* has been studied using light microscopy and scanning and transmission electron microscopy. Thread cell differentiation is remarkable in that the life history of the cell is largely dedicated to the production of a single, tapered, cylindrical, highly coiled, and precisely packaged cytoplasmic thread that may attain lengths of 60 cm and diameters approaching 1.5  $\mu\text{m}$ . Each tapered thread, in turn, is comprised almost entirely of large numbers of intermediate filaments (IFs) bundled in parallel. During differentiation of the thread, the IFs become progressively more tightly packed. Various numbers of microtubules (MTs) are found among the bundled IFs during differentiation of the thread but disappear during the latter stages of thread differentiation. Observations of regularly spaced dots in longitudinal bisections of developing threads, diagonal striations in tangential sections of developing threads, and circumferentially oriented, filament-like structures observed at the periphery of developing threads cut in cross section have led us to postulate a helically oriented component(s) wrapped around the periphery of the developing thread. The enormous size of the fully differentiated thread cell, its apparent singular dedication to the production of IFs, the ease of isolating and purifying the threads and IF subunits (see accompanying paper), and the unique position of the hagfish in the phylogenetic scheme of vertebrate evolution all contribute to the attractiveness of the hagfish slime gland thread cell as a potential model system for studying IF subunit synthesis, IF formation from IF subunits, aggregation of IFs into IF bundles and the interaction(s) of IFs and MTs.

Intermediate filaments (IFs)<sup>1</sup> and microtubules (MTs) are often found in close proximity to one another within the cytoplasm of many kinds of cells, but elucidation of specific structural and functional relationships between these two cellular components has been difficult. During earlier studies (1, 2) of the two large and distinct cell types in the hagfish (Phylum Vertebrata, Class Agnatha) slime gland, i.e., gland

thread cells (GTCs) and gland mucous cells (GMCs), it occurred to us that the GTC might serve as a useful cellular model for studying (a) IF subunit synthesis, (b) formation of IFs from IF subunits, (c) aggregation of IFs into IF bundles, and (d) the interaction(s) of IFs with MTs.

Details of the gross histological organization of the slime glands and skin of hagfishes have been reported (3–5). Differentiation of the GTC is largely dedicated to the production and packaging of a single, highly coiled, proteinaceous cytoplasmic thread within a roughly elliptically shaped cell having

<sup>1</sup> Abbreviations used in this paper: GMC, gland mucous cell; GTC, gland thread cell; IF, intermediate filament; MT, microtubule.

dimensions of  $80 \times 150 \mu\text{m}$  or more. That each thread cell manufactures one, and only one, cytoplasmic thread, and packages that thread with remarkable precision was recently demonstrated via scanning electron microscopy (1, 6). Estimates of the lengths of the threads produced by the GTCs have varied, ranging from a few centimeters (6, 7) to upwards of 60 cm (1).

Attempts to delineate the fine structure of the threads produced by the GTCs using transmission electron microscopy (TEM) have been sparse (3, 8, 9) and at times inconsistent or conflicting, particularly with respect to the possible existence, nature, and fate of filamentous and MT-like structures associated with the threads. Blackstad (3) found no evidence of filamentous subunits in threads of GTCs from the Atlantic hagfish *Myxine glutinosa*, and suggested that if filamentous subunits existed they were almost certainly smaller than 6 nm in diameter. Fan (8), on the other hand, observed distinct, parallel 12-nm filaments in developing threads of immature GTCs from the Pacific hagfish *Polistotrema stouti*,<sup>2</sup> and suggested that as thread maturation proceeded filament coalescence occurred. Finally, Terakado et al. (9) reported that developing threads in immature GTCs from two species of Japanese hagfishes (*Paramyxine atami* and *Eptatretus burgeri*) consisted of bundles of distinctly parallel filaments 9–12-nm diam, and that as thread maturation proceeded the 9–12-nm filaments gave way to 3–6-nm filaments. The 3–6-nm filaments, in turn, were stated to be composed of still thinner 1–3-nm subfilaments twisted together (unfortunately, no figures were shown that clearly demonstrated these latter observations). Blackstad (3) made no mention of MTs or MT-like structures associated with the threads in the GTCs, but he did observe longitudinal clefts in the threads. Fan (8) described transient tubular structures (30 nm in diameter) associated with the developing threads that disappeared as the threads matured and suggested that the tubular structures were formed by fusion of filaments. Terakado et al. (9) observed one to three MT-like structures within clefts in the threads at all stages of maturation.

Due to these inconsistencies and the possibility that the GTC might serve as a useful cellular model for studying the biology of IFs and IF-MT interactions, we felt that a re-examination of the fine-structural aspects of GTC maturation, as well as a continuation of biochemical studies on the composition of the thread, were in order. In this paper we re-examine the GTC in an attempt to clarify and expand our knowledge of the structural details of GTC differentiation, with a particular emphasis on the maturation of the thread contained within the GTC. In addition, we report a previously undescribed, helical component seemingly wrapped around the periphery of the developing thread. Finally, we discuss why we believe the GTC would be a good model system for studies on IFs and IF-MT interactions. In the accompanying paper (10) we describe some of the progress we have made in the isolation, biochemical characterization, and manipulation of the IF subunits associated with the thread.

Preliminary reports of the work in this paper and the accompanying paper (10) have appeared in abstract form (11, 12).

<sup>2</sup> The scientific name currently in vogue for this species is *Eptatretus stouti*, which is the species used for data presented here and in the accompanying paper (10).

## MATERIALS AND METHODS

**Animal Procurement and Care:** Hagfishes of the species *Eptatretus stouti* were obtained from Pacific Biomarine Laboratories, Inc. (Venice, CA). In the laboratory they were maintained in refrigerated 30-gallon marine aquaria containing artificial sea water reconstituted from Instant Ocean. The specific gravity of the sea water was maintained at 1.025 and the water temperature was held at  $15^\circ\text{C} \pm 1^\circ\text{C}$ .

**Light Microscopy:** 1–2- $\mu\text{m}$  sections of slime glands embedded in Epon for transmission electron microscopy (see below) were cut with glass knives, mounted on glass slides, stained with the methylene blue—azure II—basic fuchsin stain of Humphrey and Pittman (13) and photographed on a Zeiss Photomicroscope using Panatomic-X film.

**Transmission Electron Microscopy:** Immediately prior to dissection, hagfishes were anesthetized by immersing them for 20 min in sea water containing MS-222 (ethyl m-aminobenzoate methane-sulfonate) at a concentration of 70 mg/liter. Slime glands were dissected free from the skin and fixed at room temperature by immersion in a fixative solution containing 5% glutaraldehyde, 4% paraformaldehyde, 1% acrolein, 0.1 mM  $\text{CaCl}_2$ , 2.5% dimethyl sulfoxide, and 0.1 M cacodylate buffer, pH 7.4. The glands were subsequently rinsed in 0.1 M cacodylate buffer, pH 7.4 containing 4% sucrose for 1 h with changes at 15-min intervals and then postfixed in 2%  $\text{OsO}_4$  in 0.1 M cacodylate buffer, pH 7.4 containing 4% sucrose for 10 h. The glands were then rinsed three times at 20 min intervals in rinsing buffer and en bloc stained in 0.25% uranyl acetate overnight. The glands were subsequently dehydrated in alcohols and embedded in Epon 812 according to the method of Luft (14). Grey-silver sections were cut with glass or diamond knives, mounted on 300-mesh grids, stained with uranyl acetate and lead citrate, stabilized with carbon, and examined in a Philips 201 transmission electron microscope. To maximize the numbers of immature and intermediately differentiated thread cells, we frequently cut sections tangential to the surface of the gland just inside the capsule.

**Scanning Electron Microscopy:** GTCs were isolated and prepared for electron microscopy as described elsewhere (1).

## RESULTS

### General Organization of the Slime Gland

Approximately 150–200 slime glands are located along the ventro-lateral body walls of hagfishes and a cross section through one of these glands is shown in Fig. 1*a*. Each gland has a connective tissue capsule surrounded by a thin layer of skeletal muscle and connects to the epidermal surface via a single pore. Contained within each gland are the gland's two secretory cell types, i.e., the GTCs and the GMCs. Immature and intermediately differentiated GTCs are found near the periphery of the gland just internal to the gland's connective tissue capsule (Fig. 1, *c* and *d*). Larger, more fully differentiated, mature GTCs are found within the interior of the gland and may attain dimensions of  $80 \times 150 \mu\text{m}$  or more (Fig. 1*b*). GMCs occupy all the spaces within the gland not occupied by GTCs. Contraction of the skeletal muscle outside the connective tissue capsule of the gland results in the holocrine secretion of the GTCs and GMCs through the gland's pore, and the interaction of the cellular products of the GTCs and GMCs with each other and seawater results in the formation of the copious slime for which the hagfishes are so well known (15).

### Thread Cell Zonation

As differentiation of the GTC proceeds, the cell becomes roughly ellipsoidal in shape, with one end becoming somewhat pointed and the other end becoming more blunted or dimpled (Figs. 1*b* and 3*a*). At the same time, distinct zones within the confines of the GTC cell membrane become apparent. We have found it convenient to refer to these zones as (*a*) the nuclear zone, (*b*) the mitochondrial-rich zone, and (*c*) the zone of thread formation. These zones are outlined

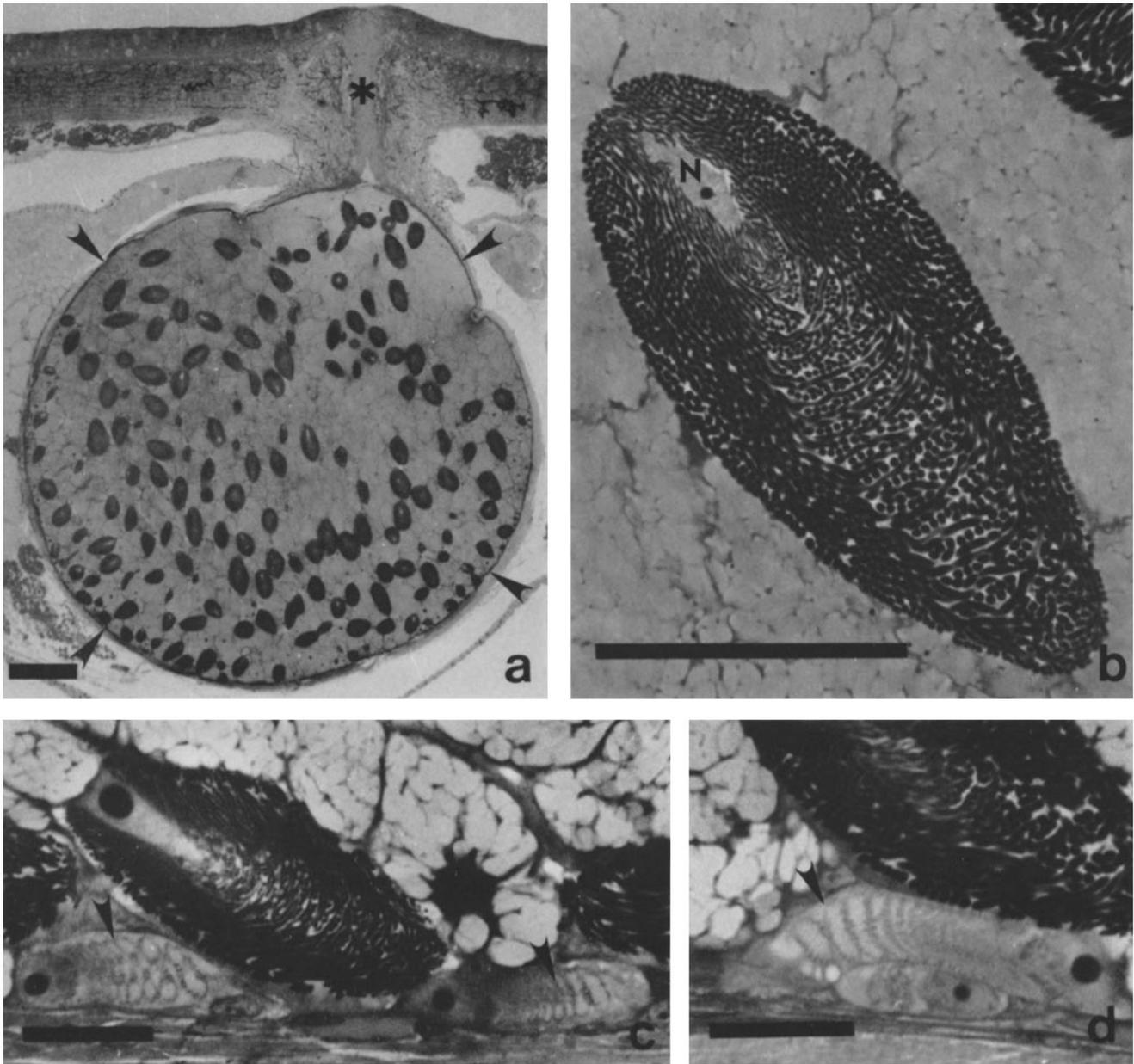


FIGURE 1 Methylene blue-basic fuchsin stained 1–2- $\mu\text{m}$  plastic sections of hagfish slime glands revealing the gland's general organization and distribution of the various stages of gland thread cell maturation within the gland. (a) Cross-section through a typical slime gland. Each gland connects to the epidermal surface via a single pore (\*). Within the gland are two types of cells, i.e., thread cells (dark staining) and mucous cells (light staining). The largest thread cells are located deep within the gland. The smaller, less differentiated thread cells are found near the gland capsule (arrowheads). (b) A relatively mature thread cell. The mature cells are invariably elliptically-shaped with a somewhat blunted end and a somewhat pointed end. The nucleus (N) is always located near the blunt end of the cell. The cytoplasm is filled with a highly coiled, intensely stained thread. Mucous vesicles of adjacent mucous cells are also apparent. (c and d) Immature and intermediately differentiated thread cells. The immature thread cells (arrowheads) lie immediately adjacent to the connective tissue capsule of the gland. The coiling of the developing thread is readily apparent. Intermediately differentiated thread cells also lie in close proximity to the capsule, but the threads are larger, appear more highly coiled, and stain intensely. Each thread cell has a single nucleus (usually containing one, but occasionally several, distinct nucleoli) situated near one end of the developing thread cell. Bars, 200  $\mu\text{m}$  (a); 50  $\mu\text{m}$  (b); 20  $\mu\text{m}$  (c and d).

(Fig. 2) in a transmission electron microscopy of an immature GTC.

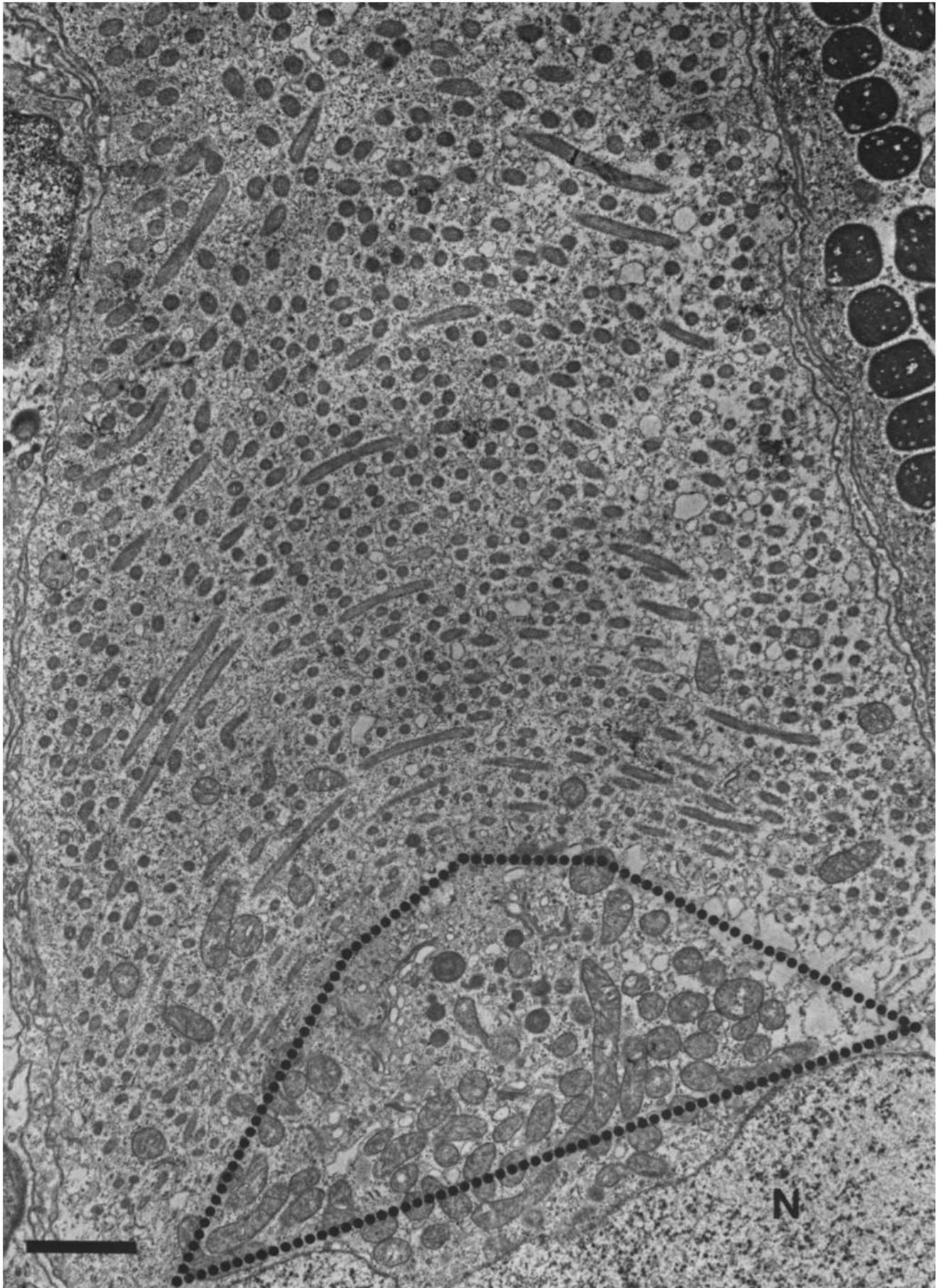
### Nuclear Zone

The nucleus constitutes the nuclear zone, and at a very early stage of differentiation it comes to occupy its eccentrically placed position near the blunt, or proximal<sup>3</sup> end of the

<sup>3</sup> Proximal, as used in this paper, refers to cellular structures or

developing cell (Fig. 1, c and d). The nucleus usually contains a large, round, electron-dense nucleolus, though occasionally two or three nucleoli may be present. The nuclear chromatin is typically dispersed with few heterochromatic regions pres-

components at, near, or toward the blunt end of the cell, whereas distal will be used to refer to cellular structures or components situated away from the nucleus toward the pointed end of the cell.



ent. The nucleus shows no remarkable changes during the differentiation process.

### Mitochondrial-rich Zone

The mitochondrial-rich zone is a conically shaped region of cytoplasm located on the distal<sup>3</sup> side of the nucleus between the nucleus and the zone of thread formation. This zone is readily identifiable because of the large numbers of mitochondria within it. In addition, this zone typically contains some Golgi figures, ribosomes, and small amounts of rough endoplasmic reticulum (Fig. 2). The base of the conically shaped mitochondrial zone lies adjacent to the nucleus, and the zone tapers as it moves distally toward the zone of thread formation. Although mitochondria are concentrated in this zone, they are not totally restricted to it, and occasional mitochondria can be found between the components of the developing thread in the zone of thread formation. As the GTC matures, the percentage of total cell volume occupied by this zone gets smaller because of the rapidly expanding volume of the zone of thread formation.

### Zone of Thread Formation

The zone of thread formation is the largest zone in the thread cell and consists of the developing thread itself and the intervening cytoplasm. This zone lies distal to the mitochondrial-rich and nuclear zones and can be visualized as a cap-shaped zone surrounding the mitochondrial-rich zone and nucleus.

**THE CYTOPLASM IN THE ZONE OF THREAD FORMATION:** The cytoplasm found within the zone of thread formation is most conspicuous in the immature GTC and is gradually reduced in size at the expense of the ever-increasing dimensions of the developing thread as the GTC matures (cf. Figs. 2, 5, and 7 with Figs. 8, 10, and 12). In the immature GTC the cytoplasm is largely occupied by ribosomes and polyribosomes, with an occasional mitochondrion and some small components of the rough endoplasmic reticulum scattered around (Figs. 4 and 5). At maturation, these cytoplasmic components have largely disappeared, and the zone of thread formation is occupied almost entirely by thread (Fig. 12).

**THE THREAD IN THE ZONE OF THREAD FORMATION:** The tapered (narrower at one end and broader at the other end), cylindrical shape of the developing thread becomes evident very early in the GTC maturation process, and since it loops back and forth through the cytoplasm in the zone of thread formation, any one section through a GTC reveals cross sections, oblique sections, and longitudinal sections of the thread that have various diameters. The narrowest portion of the tapered thread lies at the interface of the mitochondrial-rich zone and the zone of thread formation (Fig. 2). Distal and lateral to that interface region, the diameter of the thread increases progressively (Figs. 2 and 4).

As the GTC matures, there is remarkable growth of the thread, both in width and length. Evidence of increasing

thread length is most apparent when immature, intermediately differentiated, and mature GTCs are pictured adjacent to one another (Fig. 1, *b-d*). In immature GTCs the number of thread loops is relatively small and the loops are found primarily distal and lateral to the mitochondrial-rich zone. As the GTC matures, the number of thread loops (and thus length) increases dramatically, the zone of thread formation expands rapidly in all directions, and components of the thread become evident lateral and even proximal to the nucleus at the blunt (dimpled) end of the cell (Fig. 1, *b-d*). Although the dynamics of thread lengthening are not entirely clear, structural evidence suggests that lengthening occurs primarily from the narrow end of the tapered thread (see Discussion).

The GTC's singular dedication to the manufacturing and precision packaging of a single cytoplasmic proteinaceous thread, capable of unraveling to considerable lengths when secreted, make the GTC a truly remarkable cell. Although the precise packaging of the thread (1, 6) need not be described here, the end result of that packaging can be appreciated from Fig. 3.

As revealed by transmission electron microscopy, the principal structural components of the thread consist of two longitudinally oriented components, i.e., filaments and MTs, and a helical component wound around the thread periphery. Each of these components is discussed below.

**Filaments.** The bulk of the developing thread in immature GTCs (Figs. 2, 4-7) is comprised of 10-12-nm-diam filaments amassed in parallel array into a tapered, cylindrical bundle. Each filament courses for an indeterminate length along the longitudinal axis of the developing thread, and increasing numbers of filaments are found as the diameter of the tapered cylindrical bundle increases. In an immature GTC, the thread is discernible near the narrow end when as few as 6-12 filaments are bundled together (Fig. 4); near the broad end of the same thread hundreds of filaments may be bundled into a cylindrical grouping. Adjacent filaments are separated from each other by an electron-lucent space (Figs. 4-7), and this interfilament spacing is consistent within any given GTC along the entire length of its tapered cylindrical thread. However, the interfilament spacing is not consistent from one GTC to another, i.e., as GTCs and their threads mature, the interfilamentous spacing within the threads decreases. Finally, there is structural evidence which suggests that the filaments are laid down in an orderly, sequential process at the periphery of the developing thread. This is particularly evident in immature threads, where cross sections through the thread reveal filaments apparently laid down in circumferential or very gradual, outward spiraling arrays (Figs. 5, *inset* and 6).

Intermediate stages of thread cell differentiation (Figs. 8-11) are characterized by (a) massive recruitment of new filaments into the developing thread and (b) reduction in the filament-to-filament spacing within the thread. These two events lead to (a) increases in thread diameter (Fig. 2) and

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FIGURE 2 Transmission electron micrograph of a fairly immature thread cell revealing the three zones of the thread cells as delineated in the text. The nucleus (N) is found at the blunt end of the cell. A conical-shaped zone rich in mitochondria (outlined by ●●●●●●●●) lies between the nucleus and the zone of thread formation. The largest zone is the zone of thread formation, and profiles of the developing thread can be seen in this zone. It is apparent even at this early stage of differentiation that the thread is tapered, with the narrow end of the thread adjacent to the mitochondrial-rich zone. Distally (toward the pointed end of the cell away from the nucleus) and laterally, the thread gets progressively broader in diameter. Portions of a thread from an intermediately differentiated thread cell are visible at the upper right portion of the figure. Bar, 2  $\mu$ m.

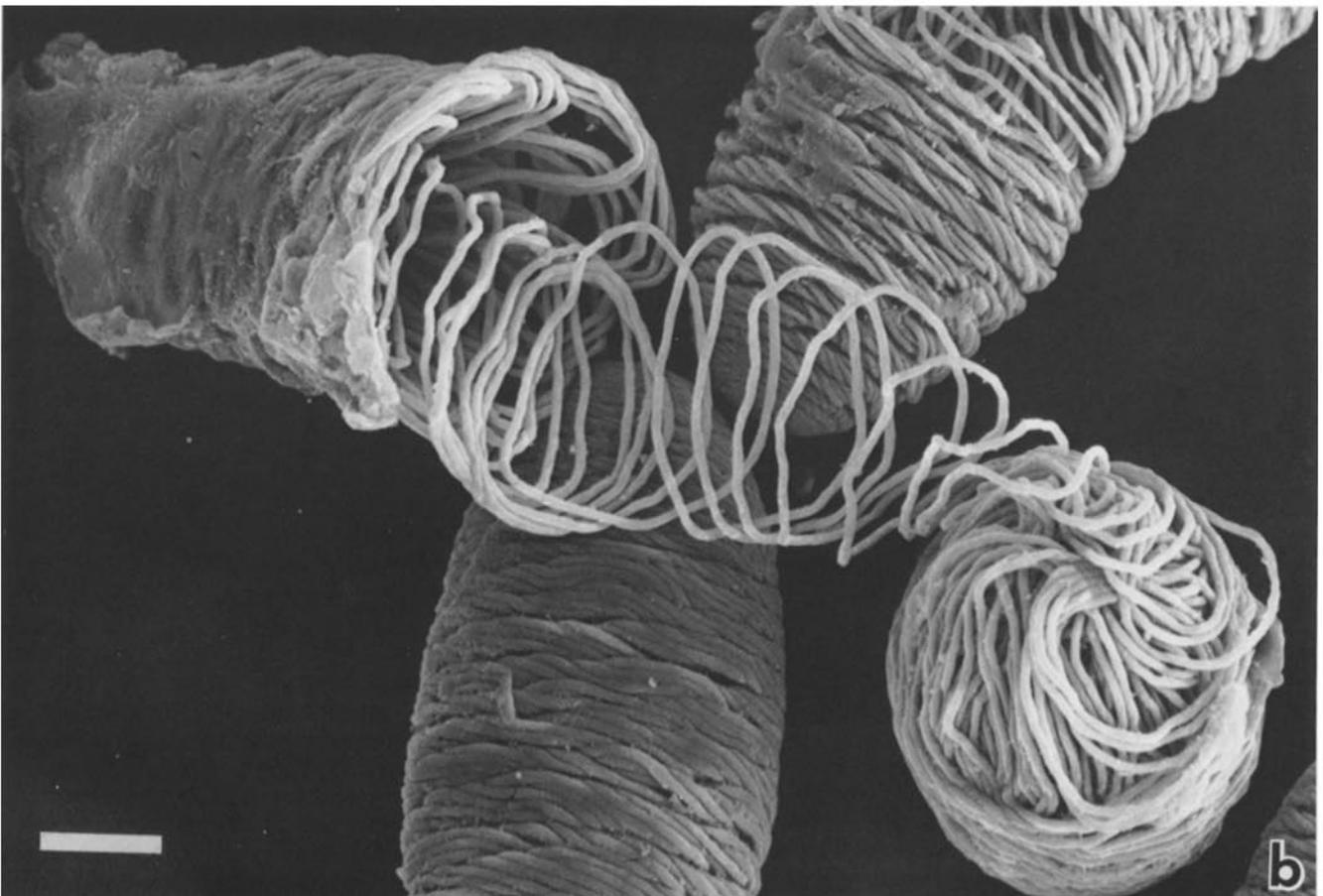
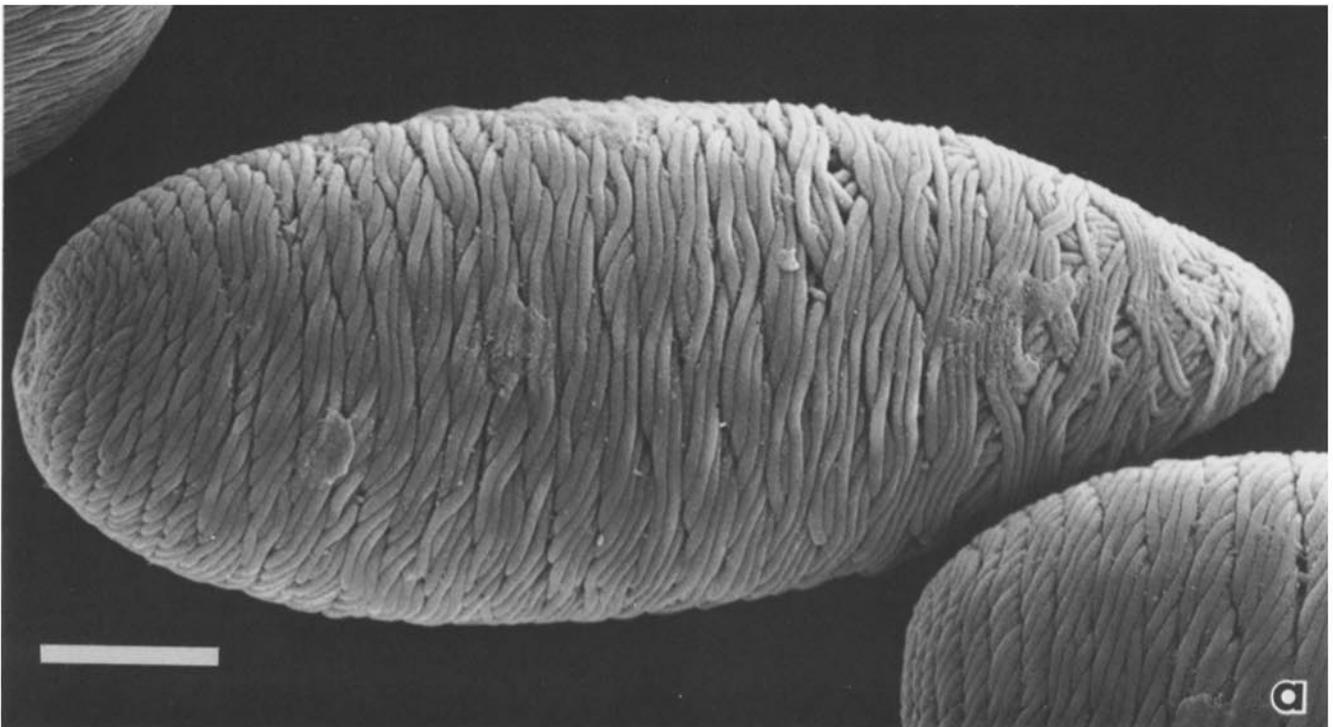


FIGURE 3 Scanning electron micrographs of two isolated thread cells. (a) An isolated thread cell without its plasma membrane. The precise packaging of the single thread contained in each thread cell is evident. (b) A thread cell that has been "pulled apart", revealing how the thread is packaged within the cell as a series of sequential loops (for details of packaging see references 1 and 6). Bars, 20  $\mu\text{m}$ .

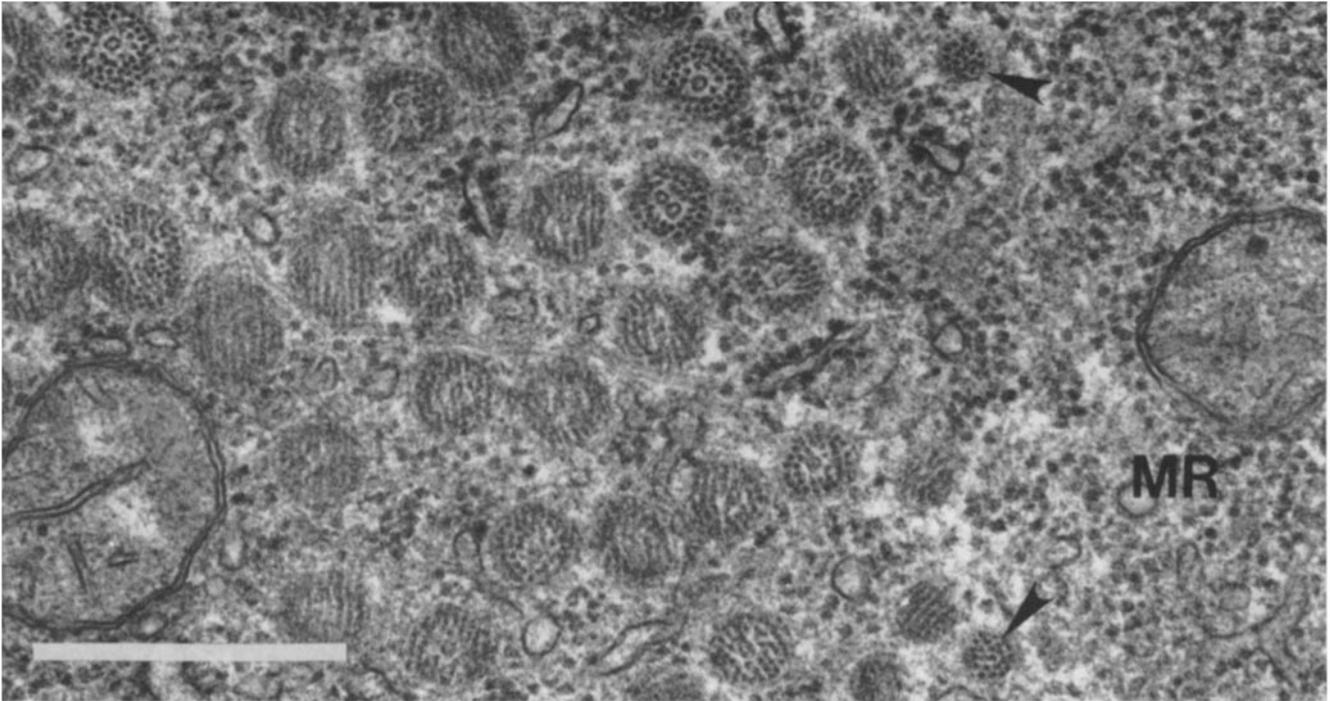


FIGURE 4 Electron micrograph of a portion of an immature thread cell near the interface between the zone of thread formation and the mitochondrial-rich zone (*MR*). The smallest portions of the thread (arrowheads) are adjacent to the mitochondrial-rich zone (*MR*); distal (toward the left of the figure) to that region the thread gets wider. It is apparent that the thread is comprised of parallel bundles of 10–12-nm filaments. Microtubules are visible in some of the cross-sections of the developing thread. Bar, 0.5  $\mu\text{m}$ .

thread length with maintenance of thread taper (see below and Discussion) and (*b*) an increase in the thread's apparent electron density. Since the interfilamentous spacing within the threads decreases (cf. Figs. 6 and 9) as the threads mature, but the diameter of the threads continually increases, there must be a massive recruitment of new filaments into the threads. Cross sections through intermediately differentiated threads near their broad ends reveal both the closer spacing of filaments as well as the massive increase in filament numbers (Figs. 9 and 10). As mentioned above, however, within any given GTC, the interfilamentous spacing remains relatively constant. Thread taper (Fig. 8) is retained and, moving from the broad end of the tapered thread to the narrow end, the number of filaments that make up the thread gets smaller. The threads in the intermediately differentiated GTCs are more electron dense than those in the immature GTCs, and this is due, at least in part, to the gradual reduction in interfilamentous spacing as the threads mature.

During the latter stages of thread maturation (Fig. 12), the filaments comprising the thread become very tightly packed together, the threads in turn become very electron dense, and the spacing between adjacent filaments is difficult to measure. Many of the filaments measure 10–12 nm in diameter, but the possible existence of filamentous components with smaller diameters cannot be eliminated. Attempts to clearly delineate and accurately measure smaller filamentous types have proved inconclusive at the present time (see Discussion).

The maintenance of thread taper, the continuing thread elongation, and the continuing increases in thread diameter as GTC maturation proceeds have, when taken collectively, important implications with respect to the dynamics of thread formation and filament recruitment. In addition, the uniformity with which filament compaction occurs along the entire

length of the developing thread (simultaneous rather than wave-like) may have implications with respect to maturational processes possibly occurring within the thread (see Discussion).

*MTs.* A second component found in the developing thread is the MT. These structures, which measure  $\sim 25$  nm in diameter and run for indeterminate lengths, lie parallel to the filaments, coursing in the direction of the longitudinal axis of the developing thread. The number of MTs present in any particular portion of the tapered thread is quite variable, which indicates that individual MTs do not run the entire length of the developing thread. Proceeding from the narrow end to the broad end of the tapered thread, the number of MTs seen in any particular cross section of the thread tends to increase. At the interface of the mitochondrial-rich zone and the zone of thread formation, where the narrowest portions of the developing thread are seen, the number of MTs within the thread typically varies from zero to two (Fig. 4). As one moves distally and/or laterally to regions of increasing thread diameter, the numbers of MTs increase, and in the immature GTC it is not uncommon to see broader sections of the thread containing 8–10 MTs randomly scattered among the filaments; where numerous MTs are observed they may occur singly or in small clusters of 2–4 MTs (Figs. 5 and 6).

In intermediately differentiated GTCs, the numbers of MTs increase dramatically, and it is not uncommon to see 20 or more MTs in cross sections through the thread near its broader end (Fig. 9). Typically, MTs in the intermediately differentiated GTC occur singly or in groups of two to four, but larger groupings, e.g., seven to eight MTs, are common (Fig. 9).

In immature GTCs, the filament-to-filament spacing and filament-to-MT spacings are wide enough to make a zone of exclusion not readily apparent around the MTs. However, as

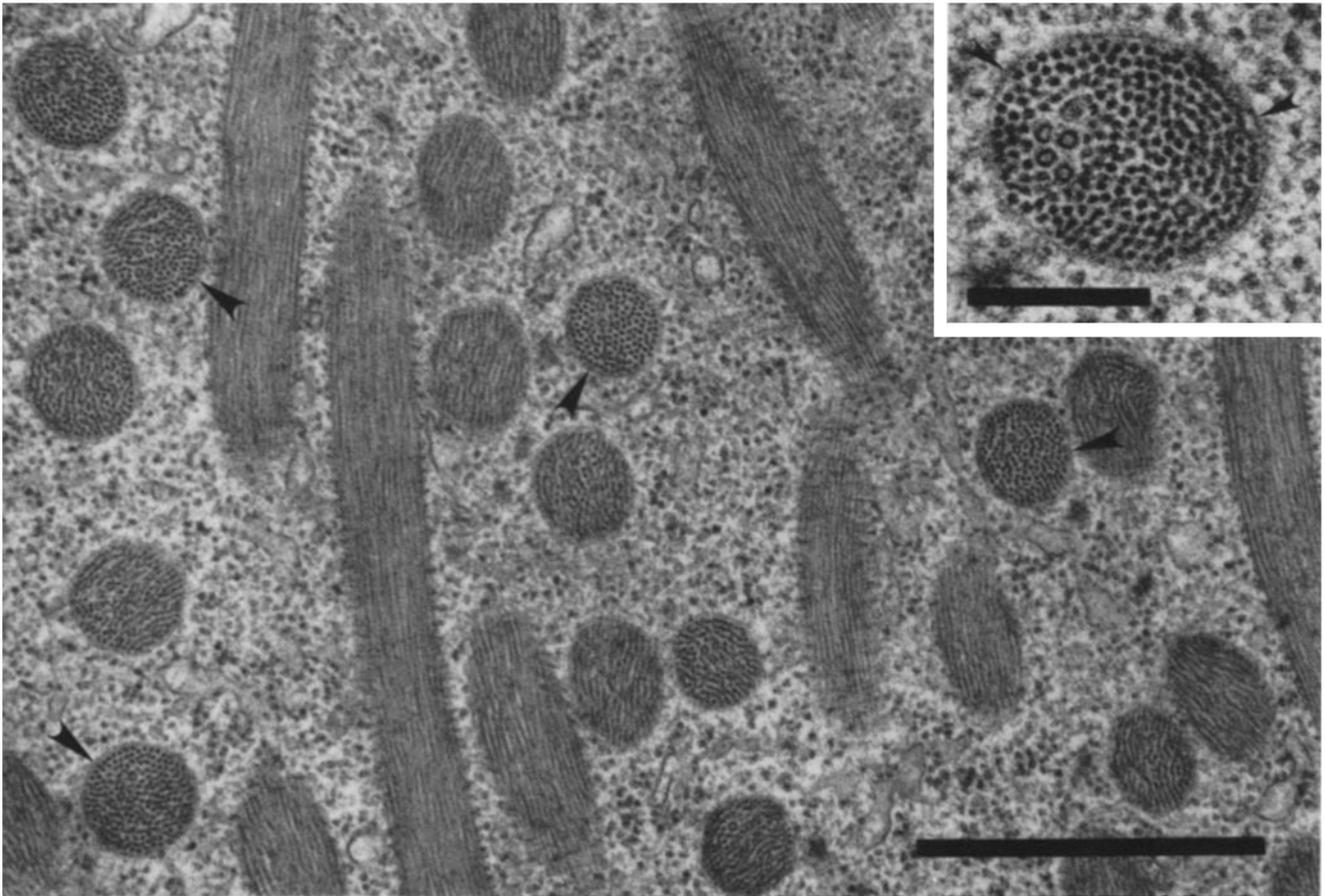
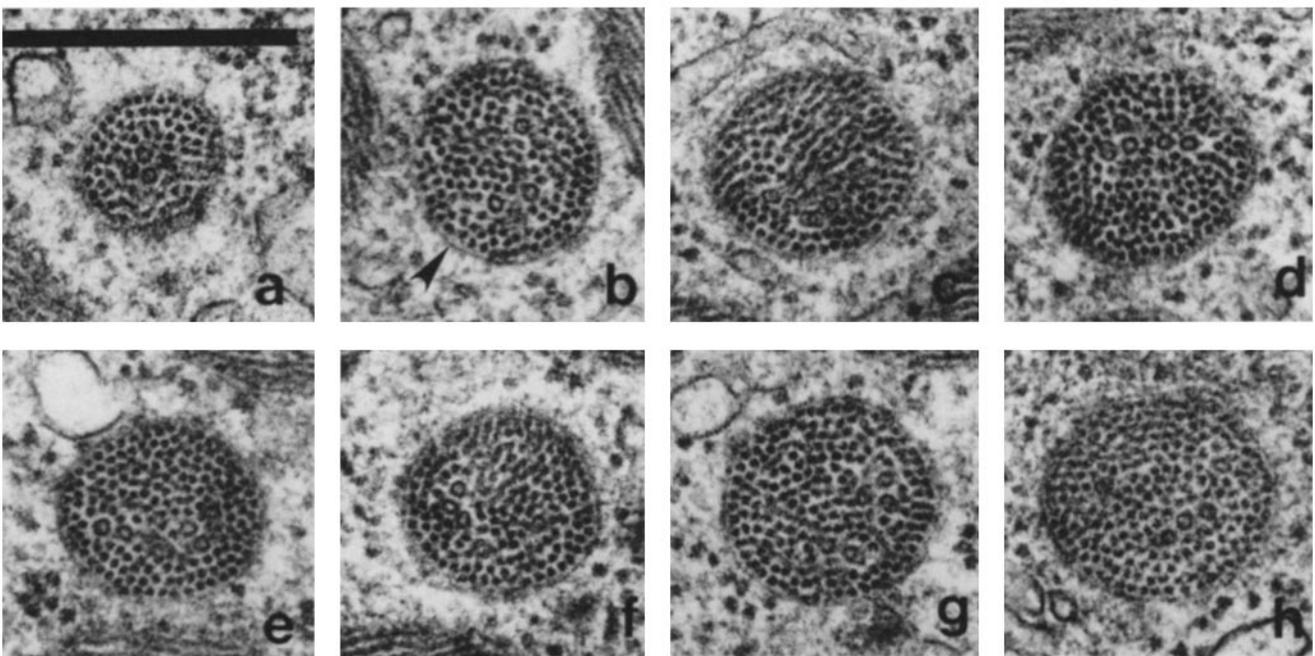


FIGURE 5 Electron micrograph of a fairly young thread cell revealing both longitudinal and cross-sections of a single developing thread in the zone of thread formation. In many of the cross-sections of the thread a peripheral, circumferentially oriented electron density is visible (arrowheads). The individual IFs are readily identifiable, with distinct spaces existing between adjacent filaments. MTs are apparent in some of the thread profiles. The cytoplasm between the thread profiles is largely filled with ribosomes. (*inset*) Electron micrograph of a cross-section through a portion of a developing thread in a young thread cell. Four to five microtubules are evident within this bundle of 10–12-nm filaments. Particularly evident is the tendency of the filaments to appear in concentric rings or patterns (see also Fig. 6). There is also a circumferentially oriented electron density (arrowheads) at the periphery of the IF bundle. Bar, 1.0  $\mu\text{m}$ ; 0.25  $\mu\text{m}$  (*inset*).



the thread in the GTC matures, the filament-to-filament spacing within the thread is reduced while the filament-to-MT spacing is not, and a zone of exclusion, halo, or cleft surrounding the MTs or MT clusters becomes more apparent

(Figs. 9–11).

The MTs almost always appear internalized within the bundle of filaments when associated with the developing thread; i.e., they are rarely found at the edge of the bundle of

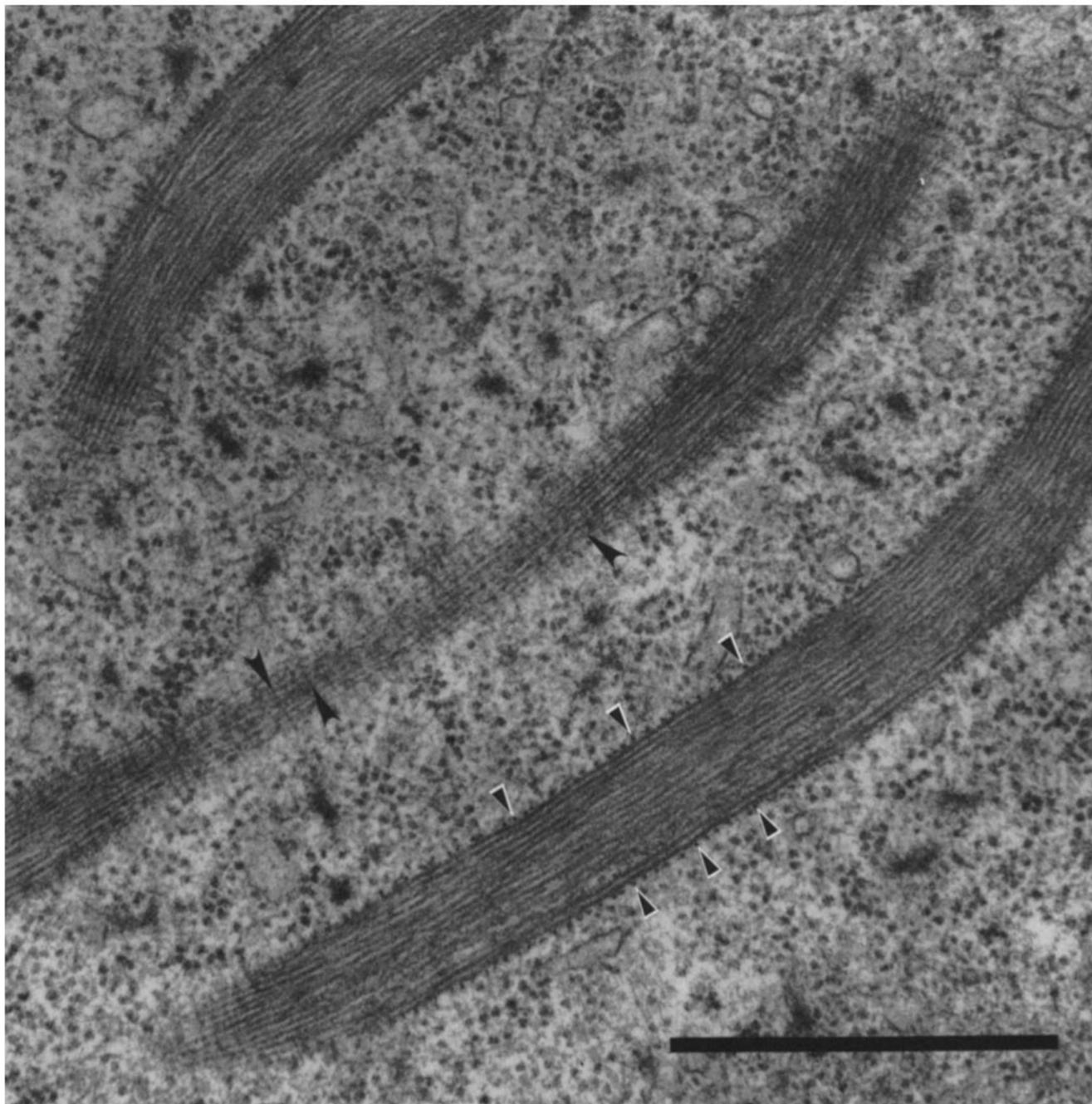
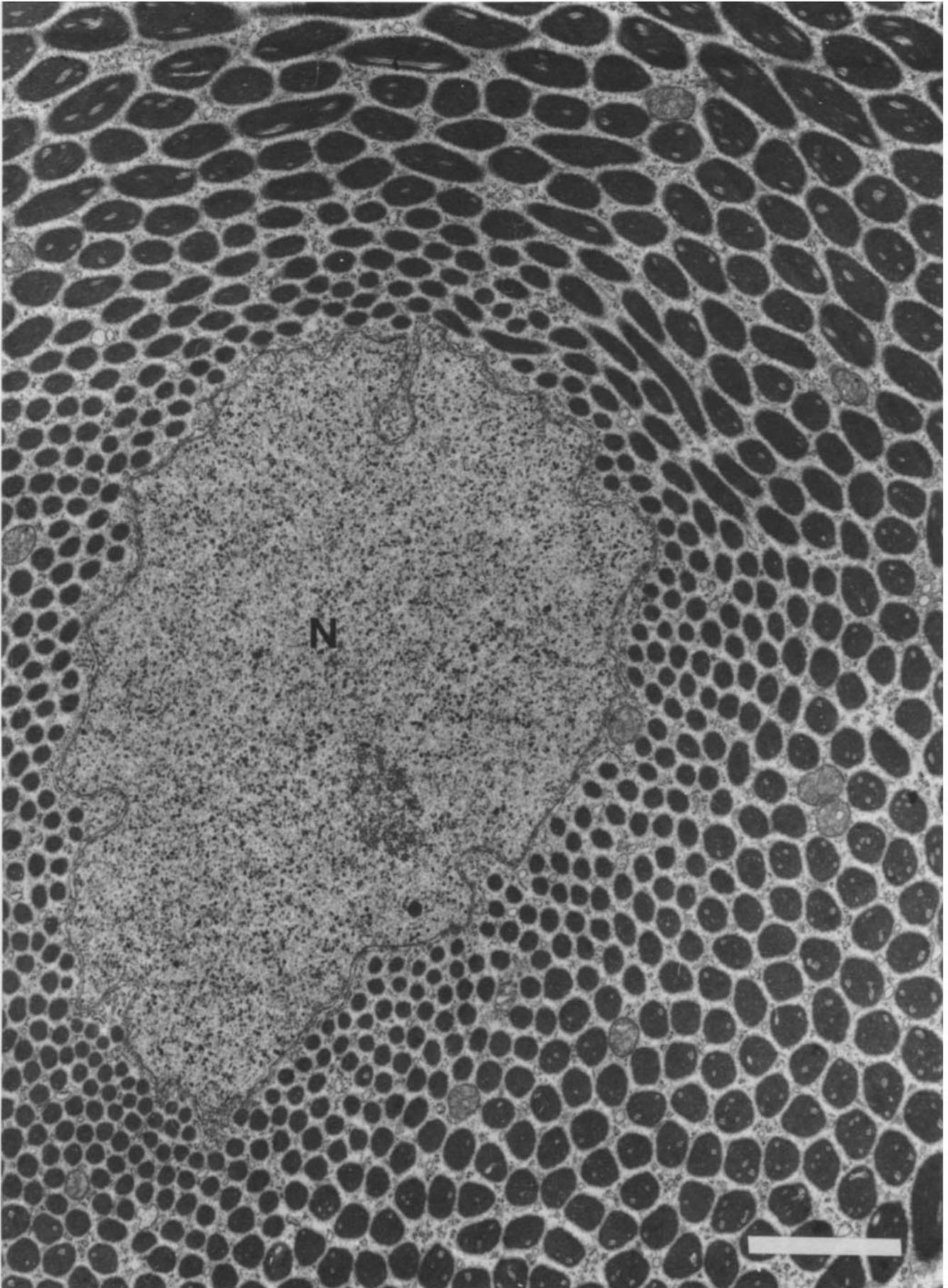


FIGURE 7 Electron micrograph revealing longitudinal sections through a developing thread. In the longitudinally bisected thread at the lower right of the figure a number of evenly spaced dots (small arrowheads) are seen at the surface of the bundle of IFs. The dots are  $\sim 36$  nm apart. In the tangentially cut thread in the middle of the figure cross-striations (large arrowheads) can be seen crossing the IF bundle almost, but not quite, perpendicular to the IFs, i.e., the striations appear slightly pitched away from the true perpendicular (compare Fig. 11). The visualization of a circumferentially-oriented electron density in many cross-sections of developing threads, and regularly spaced dots or striations in bisections or tangential sections of longitudinally oriented threads respectively, have led us to postulate the existence of a helically-oriented component situated or wrapped around the developing bundle of IFs and MTs comprising the thread. Bar,  $1.0 \mu\text{m}$ .

FIGURE 6 Selected electron micrographs of cross-sections through relatively immature threads, all of which reveal the tendency of the IFs to be arranged in distinct concentric rings (or spirals). In most of the figures a circumferentially-oriented electron density is apparent (particularly in *b* [arrowhead]) at the periphery of the cylindrically bundled IFs. Bar,  $0.5 \mu\text{m}$ .



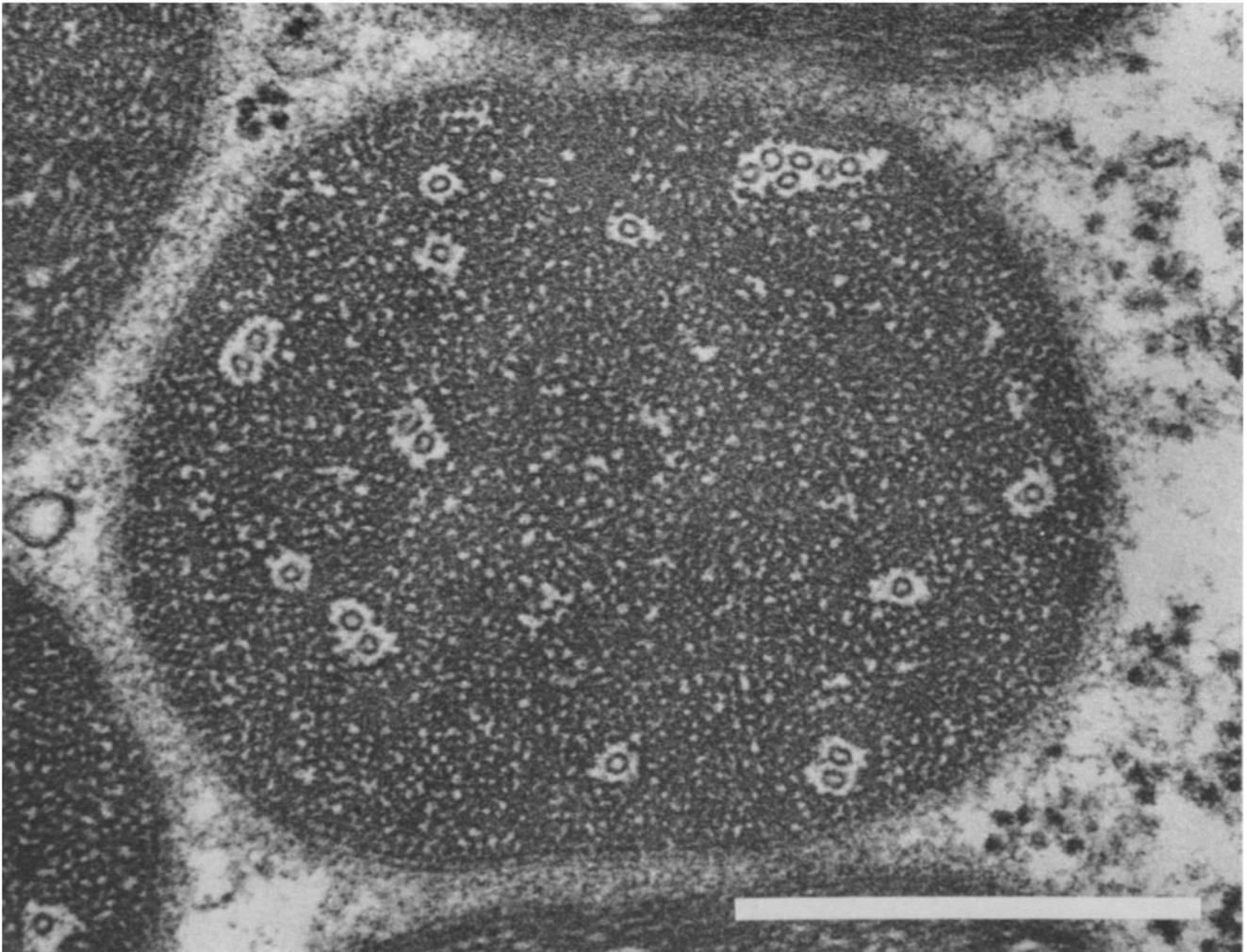


FIGURE 9 Electron micrograph of a cross-section through a developing thread in an intermediately-differentiated thread cell. When compared with the IFs in an immature thread, the IFs in an intermediately differentiated thread are closer together and make the thread look more electron dense. As the thread gets wider, the number of MTs in any one cross-section tends to increase. In this particular profile 21 MTs are apparent and occur singly, in twos, and in one large cluster of six. Surrounding the MTs is an electron lucent space or halo which we refer to in the text as the exclusion zone. Bar, 0.5  $\mu\text{m}$ .

filaments. Since we have been unable to determine precisely the absolute end of the developing thread (presumably the narrowest end), it has been impossible to determine whether MTs or filaments appear first.

As the threads reach maturity (Fig. 12), there is a gradual loss of MTs along the entire length of the thread, and at maturity there are practically no MTs detectable in cross sections through the thread profiles. In addition, the large halos, or clefts, in which the MTs were observed in earlier stages of differentiation also disappear, and there remains little evidence of MTs ever having been associated with the developing thread.

**Helical Component.** At the periphery of the immature and intermediately differentiated thread, a filament-like com-

ponent can be visualized that is seemingly wrapped around the longitudinally bundled IFs and MTs that make up the bulk of the thread. The orientation of the peripheral component is such that it is not quite perpendicular to the longitudinal axis of the thread, but is at a slight angle to the perpendicular. Structural evidence for the helical component comes from three different types of sections through the developing threads: (a) tangential sections through the surface of the thread, which reveal diagonally spaced striations (Figs. 7 and 11); (b) sections that bisect the thread longitudinally, which reveal evenly spaced dots at the surface of the developing thread (Figs. 7 and 11); and (c) cross sections of the threads, which reveal circumferentially oriented electron densities at the thread periphery (Figs. 4–6). In the immature thread cells,

FIGURE 8 Electron micrograph of an intermediately differentiated thread cell. With the exception of the nucleus (N), the bulk of the cell is filled with sectioned profiles of the developing thread. This particular section does not pass through the mitochondrial-rich zone, which would be above (or below) the nucleus. The tapered nature of the developing thread is obvious, with the smallest profiles lying closest to the nucleus. Moving laterally away from the nucleus the profiles get progressively wider. At this magnification, distinct IFs and MTs are not readily apparent, but the spaces or clefts that tend to occur in the broader thread profiles contain one or more MTs. The electron density of the thread is greater than that of the immature thread due to a tighter packing of the IFs comprising the thread. Bar, 2.0  $\mu\text{m}$ .

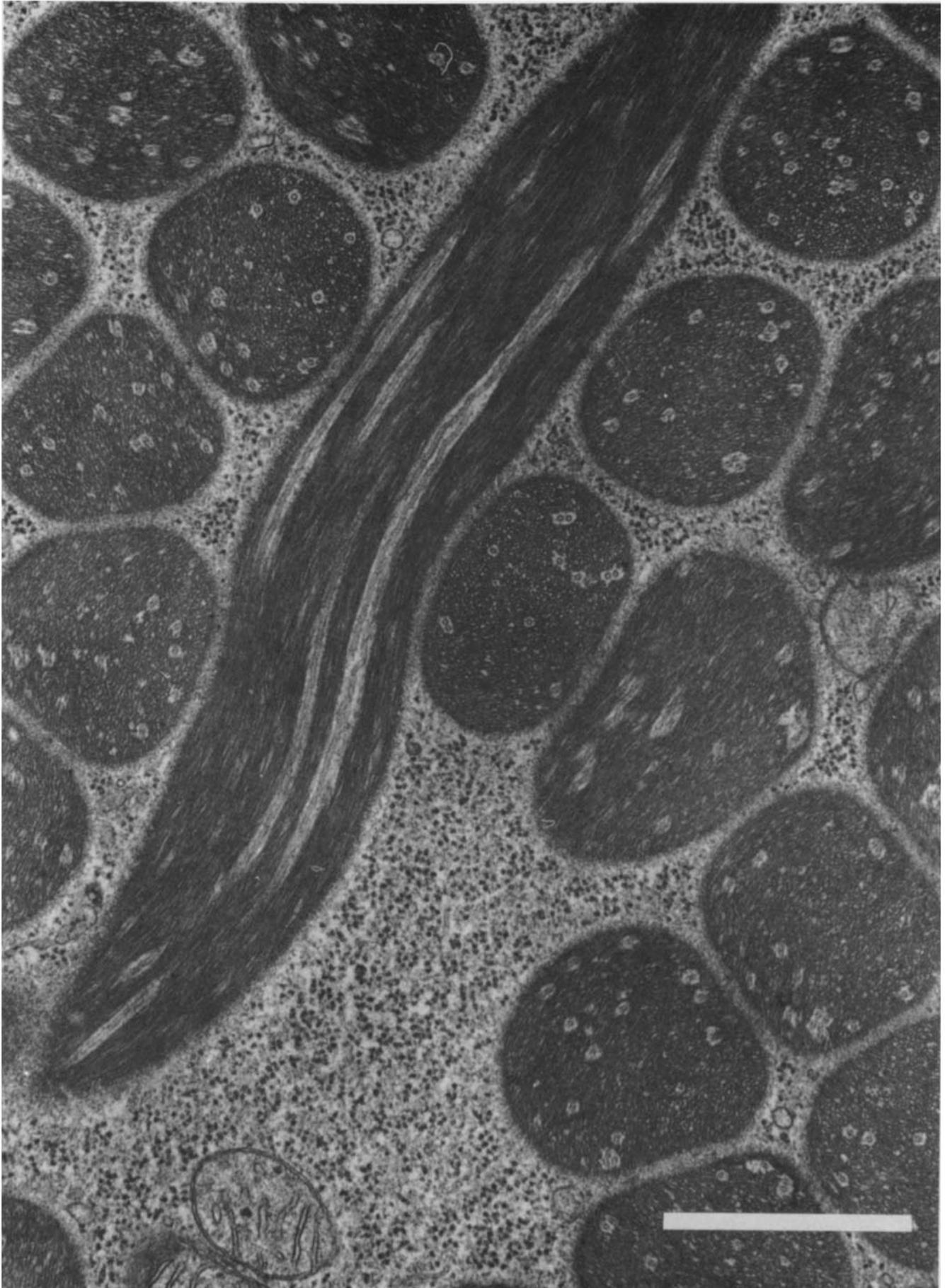
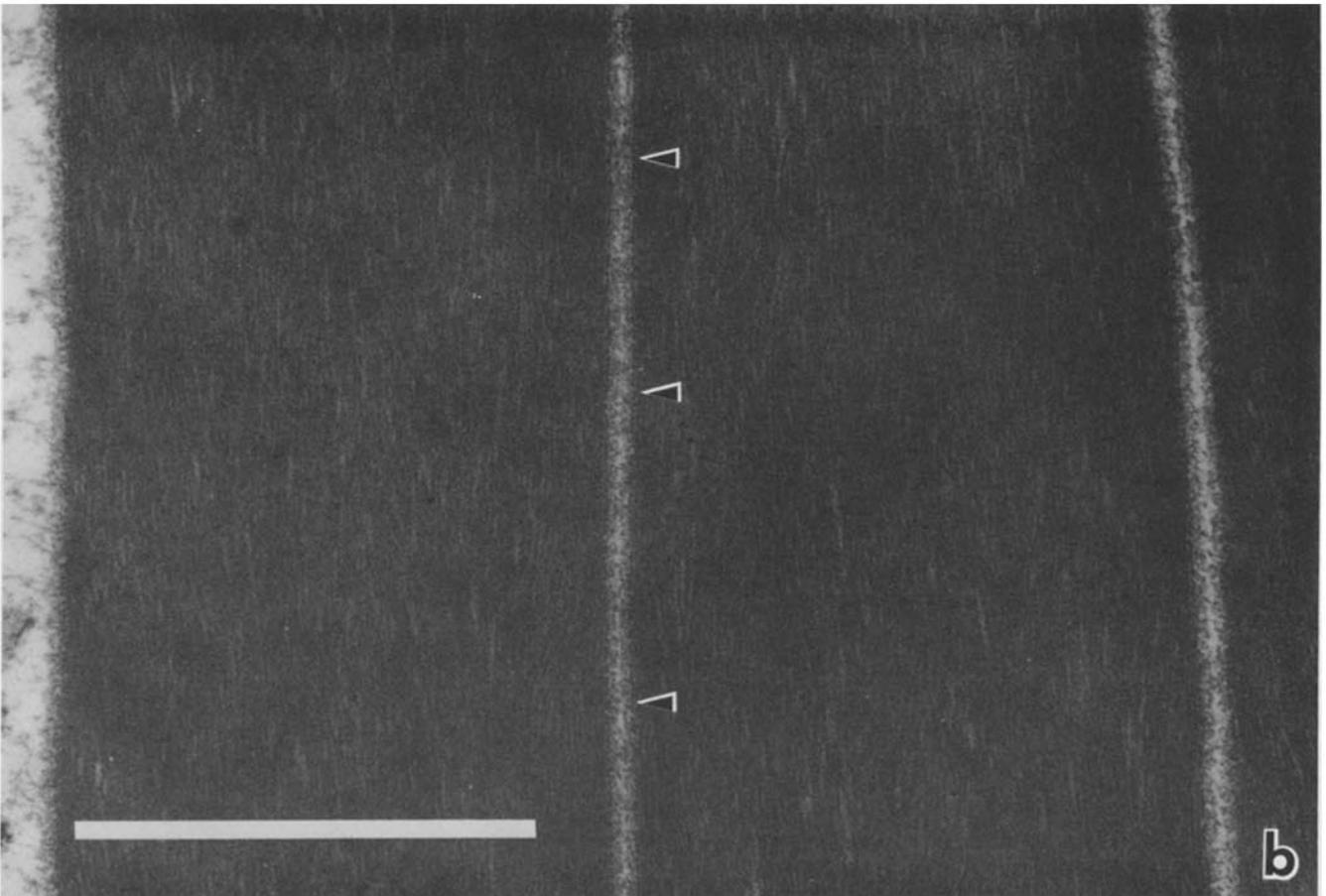
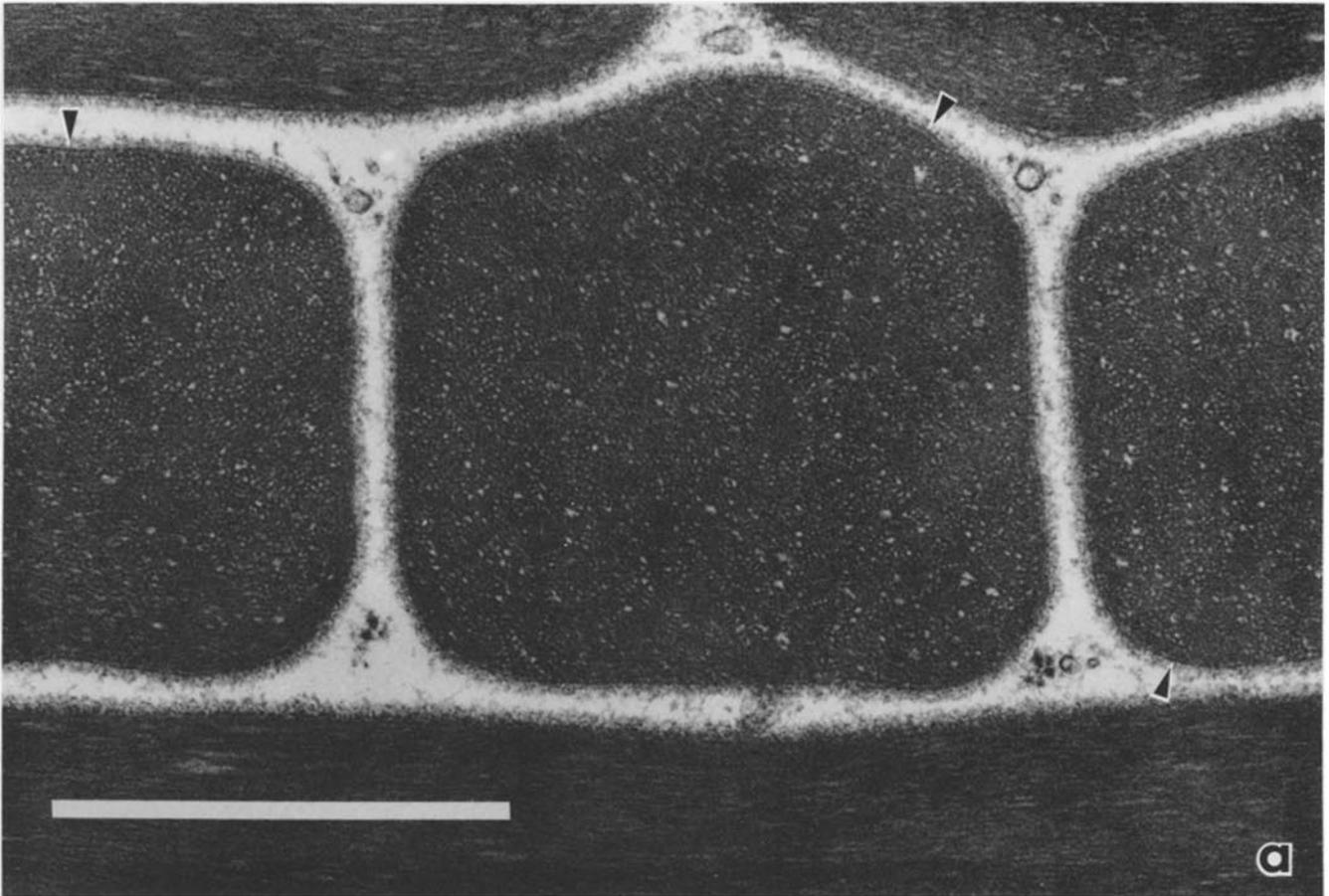


FIGURE 10 Longitudinal and cross-sections of an intermediately differentiated thread. MTs are seen running parallel to the IFs within their clefts or halos. The tremendous increase in the numbers of IFs comprising the intermediately differentiated threads when compared with the numbers seen in the immature thread is apparent. Bar, 1.0  $\mu\text{m}$ .



FIGURE 11 Longitudinal profiles of an intermediately differentiated thread. Where the thread is cut tangentially, pitched striations can be seen crossing the bundles of IFs. In addition to MTs occurring within the clefts of the thread, there are occasional MTs lying free in the cytoplasm (arrowheads). Bar, 1.0  $\mu\text{m}$ .



the spacing of the striations and dots in the longitudinal, tangential, and bisected views, respectively, is ~36 nm. The fact that in cross sections of the thread the circumferentially oriented densities do not pass completely around the bundle of IFs and MTs (see, e.g., Fig. 6) is further evidence of its helical arrangement; i.e., the helical component will be in the focused plane of section for only a part of its path around the filament bundle. The helical component is difficult to visualize in the final stages of maturation, although suggestions of a peripheral component (Fig. 12) remain. In cross sections through the threads (Fig. 12*a*), a circumferentially oriented structure is apparent, and bisections of the thread along the thread's longitudinal axis (Fig. 12*b*) reveal very closely spaced dots at the thread surface. In addition, there is a fuzzy or flocculent-looking material associated with the thread surface (Fig. 12, *a* and *b*).

## DISCUSSION

### *Justification for Calling the GTC Filaments IFs*

IFs are cytoplasmic fibrous structures that measure 7–12 nm in diameter, are of indeterminate length, and are formed by the interaction of chemically heterogeneous, proteinaceous subunits (16). On the basis of physical measurements, the filaments that make up the bulk of the threads in the hagfish GTCs thus fall into the IF category rather than the thin actin filament (6 nm) or thick myosin filament (15 nm) category. In addition, biochemical data indicate that, like other IFs, GTC IFs are comprised of proteinaceous subunits (1, 10, 12). Furthermore, of the various classes of IFs now recognized (16), it appears that the hagfish GTC IFs are most similar to the class of IFs referred to as keratin (tono) filaments found in epithelial cells or cells of epithelial origin. Evidence supporting this classification of hagfish GTC IFs includes the facts that the GTC is an epithelially derived cell, that the molecular weights of the polypeptide subunits (i.e., 63,500) are within the range of molecular weights found for other keratin (tono) filament subunits (16), and that the polypeptide subunits show reasonable similarities in amino acid composition to other keratin (tono) filament proteins (10). The molecular weights of the GTC IF subunits (10) are considerably higher than those found for avian feather keratins (17–19).

### *Dynamics of Thread Formation*

Although a number of morphological and biochemical studies on various aspects of GTC biology have been done (1, 3, 4, 6–9), only Newby (7) attempted to explain how the thread within a GTC was formed. Observations on GTC differentiation, made possible by the advent of TEM and SEM techniques after Newby's paper, necessitate reconsideration of the dynamics of thread formation. The hypothesis that we present here attempts to take into account the following morphological observations related to thread differentiation over a period of time: (*a*) the apparent increase in thread length, (*b*) the increase in thread diameter, (*c*) the maintenance

of thread taper, and (*d*) the precision packaging of the thread within the GTC cytoplasm.

We suggest that very early in the differentiation of a GTC, a number of intermediate filaments line up parallel to one another to form an initial, relatively short, narrow bundle of filaments which we will refer to as the primal thread. Once formed, the primal thread serves as a nucleation or deposition site for all subsequent IF subunits synthesized within the cytoplasm of the GTC. Although three possible sites of IF deposition on the primal thread exist, i.e., at either of the two ends or laterally, we propose that IF additions at one of the two ends (i.e., the distal end) is somehow blocked, and as such, all new IF subunits synthesized are incorporated either laterally or at the unblocked (proximal) end. Continuing IF subunit deposition thereafter at both accessible sites, i.e., laterally and at the proximal, unblocked end, would result in the formation of a thread that is simultaneously lengthening, increasing in diameter, and maintaining taper. The blocked end of the mature thread thus winds up being the oldest portion of the thread. Since the blocked end is available for lateral IF deposition for the longest period of time, it also becomes the broadest portion of the tapered thread. Continuing toward the proximal end of the thread, the thread becomes newer, IF subunits are added laterally for correspondingly shorter periods of time, and the thread gets progressively narrower.

Such a hypothetical mechanism of thread formation is also compatible with the final precision packaging observed within mature thread cells (1, 6). As the thread lengthens, it must lengthen either as a long, straight rod (which does not occur) or it must coil (which does occur). If a semiflexible rod could be continually extended within a relatively confined space, the rod would tend to form loops, one upon the other, within the confines of its compartment. An analogy here would be useful: if one were to push a long garden hose into a barrel, the hose would tend to form a series of loops, with each new loop coming to lie adjacent to the previously formed loop (and this is precisely the way the thread is organized within the confines of the plasma membrane), forming a series of successive loops, each loop lying adjacent to the previous loop (for details of the packaging see references 1 and 6). It is apparent from electron micrographs (see, e.g., Fig. 2) that the proximal (narrower) end, we suggest, newer end of the tapered thread is adjacent to the mitochondrial-rich zone. Thus, as newly formed thread is generated at the interface of the mitochondrial-rich zone and zone of thread formation, it forms loops that are pushed into successively older loops that lie distal and lateral to that region. Indeed, a sectioned thread cell (Fig. 1*b*) indicates that the widest (and presumably oldest) loops or segments of the thread are those that lie most distal and lateral from the interface of the mitochondrial-rich zone and the zone of thread formation.

### *Fate of the IFs*

It is clear that as maturation proceeds, the spacing between adjacent parallel IFs within the developing thread gets smaller,

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FIGURE 12 (*a*) Cross- and longitudinal sections through a thread in a fairly mature thread cell. The IFs are very tightly packed together, the MTs are gone or greatly reduced in number, and the clefts or halos have disappeared. Very little cytoplasm exists between adjacent profiles, and where it is present it looks empty or washed out. Evidence of a peripheral helical component is still present (arrowheads). (*b*) Electron micrograph revealing longitudinal profiles of a thread in a fairly mature thread cell. The IFs are very tightly packed and the thread is very electron dense. The closely spaced dots at the thread surface (arrowheads) may represent the helical component that is more obvious in less mature threads. Bars, 1.0  $\mu\text{m}$ .



FIGURE 13. Drawing of a developing thread that attempts to account for some of the observations made in transmission electron micrographs. The thread is tapered and comprised primarily of parallel arrays of IFs and some MTs (in developmental stages). The appearance of circumferentially oriented electron dense structures in cross sections of threads (see Figs. 4–6), slightly pitched cross-striations in tangential sections of longitudinally-oriented threads (Figs. 7, 11), and regularly spaced dots at the surface of longitudinally bisected threads (Fig. 7) can be explained if one postulates the existence of a filamentous-like structure wrapped around the periphery of the IF-MT bundle in a helical manner. The nature of this helical component(s) is not clear, but we believe it is important and related to the orderly assembly of IF subunits into IFs at the periphery of the developing thread.

and that toward the end of thread differentiation, the ability to resolve individual 10-nm IFs within the thread is often difficult. Indeed, the observations of several investigators (3, 8, 9, 11) indicate that 10-nm filaments do not exist in the mature thread. Our findings conflict with these earlier observations, and we believe that at least in some instances resolvable 10-nm filaments persist even in the fully mature thread. However, we are uncertain whether, during the final stages of thread maturation, modification or rearrangement of IF subunits occurs to form smaller-diameter thin (3–6 nm) or sub-(1–3 nm) filaments similar to those reported by Terakado et al. (9). One of the difficulties that must be faced when attempting to delineate 3–6-nm and 1–3-nm filaments in sectioned material stained with uranyl acetate and lead citrate is whether one is measuring artifacts (e.g., stain particles) or real structures. Nevertheless, the possibility exists that as thread maturation proceeds, a rearrangement of the IF subunits does occur, giving rise to filamentous structures of dimensions different from those initially observed in the early stages of thread formation. Indeed, there is evidence (20, 21) that 10-nm IFs are made up of smaller protofilaments and protofibrils. If the GTC IFs are made up of smaller protofilaments and protofibrils, it is conceivable that environmental changes in the intra-thread regions (e.g., ionic changes), facilitated perhaps by MTs (see below), may permit or bring about a rearrangement of IF subunit proteins to form filamentous structures smaller in diameter than the typical 10-nm filament. If that is true, then the hagfish thread cell could prove to be useful for studying not only IF subunit assembly but also those factors that can affect and/or alter the nature of the assembled state (e.g., phosphorylation).

### Role of MTs

The significance of the MTs with respect to thread differentiation is not clear. It is tempting to say that the MTs serve merely as a temporary scaffolding around which the IF subunits aggregate or align to form 10-nm IFs, and that once the thread is well developed, the need for the MT scaffolding is lost and the scaffolding is discarded (22). The inconsistent number of MTs and their irregular or random positions within the bundle of IFs comprising the thread suggest that precise spatial positioning of the MTs within the thread is not essential for proper thread differentiation and IF recruitment. Nevertheless, the large number of MTs observed toward the broader end of the intermediately differentiated thread suggests that they do play an important role in thread maturation. It seems possible they may have more than one function, i.e., perhaps they serve a structural (e.g., scaffolding) as well as a physiologic function, such as providing intra-thread channels or pathways within the differentiating thread for changes in

ionic composition, cytoplasmic removal of material from between the IFs, etc. The fact that with time the spacing between IFs along the entire length of the thread decreases, and that this occurs not in wave-like fashion but rather simultaneously throughout the thread, suggests coordinated types of intra-thread changes or modifications, and the MTs may be implicated in the facilitation of those processes. The fact that the MTs disappear in the terminal stages of thread differentiation suggests that their function(s) is no longer necessary.

The cleft, or halo, that appears around the individual MTs or MT groups probably represents the well-documented exclusion zone commonly associated with cytoplasmic MTs (23–28). Although the function of this zone is poorly understood, the differentiating thread cell may provide a unique opportunity to examine the nature of this zone more completely. Currently, we are attempting to stain this zone using lanthanum and other reagents with the hope of demonstrating the existence or non-existence of a structure(s) that may be associated with this zone and might possibly be involved in direct IF-MT interactions. Green and Goldman (29) recently reported some experimental work which strongly suggests an association between MTs and IFs in cultured cells and indicates that the organization and distribution of IFs are dependent upon a normal cytoplasmic MT complex.

### The Helical Component

The precision with which the thread is manufactured and packaged and the taper that the thread exhibits suggest that the IF subunits are synthesized and incorporated into the developing thread in a very regular, controlled manner. Although at present we do not know how the addition of IF subunits is accomplished or controlled with such precision, we are intrigued by the helical component, which appears to be regularly spaced and helically wound around the IF bundle that makes up the developing thread (Fig. 13). We initially suggested (11) that the helical component might be involved in the bundling together of the parallel IFs into a single thread. While we still hold that function as tenable, we believe that the helical component may also be directly involved with or related to the organization and/or deposition of IF subunits at the thread periphery. Indeed, it is conceivable that the helical component consists of IF subunits that are lining up at the thread periphery immediately prior to their incorporation into the thread.

The observation of concentric, circumferential uniformity with respect to IF spacing, which is particularly apparent near the thread periphery (Fig. 6), also suggests that the IFs may be laid down sequentially, each new IF appearing adjacent to the previously formed one but one step further around the thread periphery. Toward the narrow end of the tapered

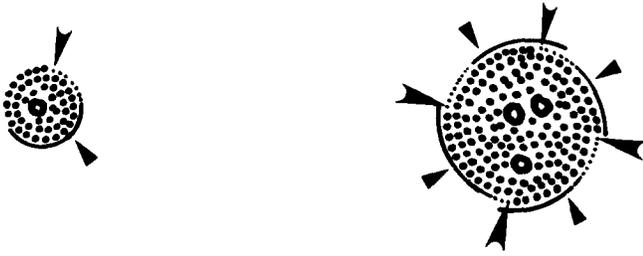


FIGURE 14 Hypothetical drawing of a cross-section through an immature thread near its narrow (left) and broad (right) end. We believe the helical component (small arrowheads) is related to the lateral deposition of new IFs at the thread periphery, and that formation of new IFs at the thread periphery proceeds in a precise, circumferential sequence. In the narrower portions of the thread, only a single helical component would be present in any one cross-section, and the next IF to be formed would appear at the large arrowheads. Toward the broader end of the thread several adjacent helical components would appear in any given cross-section, and thus several IFs would be initiated and/or assembled simultaneously around the thread periphery, as indicated by the arrowheads. Such a process would help to explain the precise circumferential relationships of adjacent IFs as drawn here and as observed in Figs. 5 (inset) and 6.

thread there may be only one or two sites at the thread periphery where IFs are formed at any one time, whereas near the broad end there may be numerous sites of simultaneous IF formation equally spaced around the thread periphery (Fig. 14).

### The GTC as a Potentially Useful Model System for Studying IFs

The GTC comes about as close to being an "IF machine," i.e., a cell dedicated almost exclusively to the manufacturing of IFs, as one might expect to find in nature. The enormous size of the mature GTC and the GTC's ability to produce millions of IF subunits, assemble those IF subunits into 10–12-nm IFs, and, in turn, bundle the IFs into a single cylindrical thread that may reach 60 cm or more, make this a truly remarkable cell. The singular dedication of the GTC to IF production should make this cell a useful system for purposes of investigating the synthesis of IF subunits, the assembly and control of assembly of IF subunits to form 10-nm IFs, formation of IF bundles, arrangement and rearrangement of IFs and IF subunits during differentiation processes, and the interaction(s) and/or relationship(s) of IFs and MTs.

For biochemical studies a definite appeal of this system is the ability to isolate large (gram) quantities of the IF subunit proteins (12) for subsequent manipulation, reassembly studies, antibody production, sequence work, etc. Amino acid sequence work on the GTC IF subunit proteins may be of considerable interest because of the unique position of the hagfish in the vertebrate scheme of evolution. Since the hagfish represents one of the most primitive living vertebrates, sequencing the IF subunit proteins isolated from the GTCs (12) may provide insights into the question of whether there are both constant (conserved) and variable (non-conserved) amino acid sequences in some, if not all, IF proteins. For example, it has been postulated that there may be constant (conserved) amino acid sequences in IF protein subunits that are responsible for the common physical and biochemical properties of IFs from many different cellular sources (30), whereas highly variable regions within the same IF subunit proteins may account for the diversity of functions and prop-

erties of IFs from one cell type to another. In support of this hypothesis are the recent findings of conserved portions of the IF proteins vimentin and desmin in cells from fishes through man (31).

In summary, it is hoped that the observations made here and in the accompanying paper (10) will make apparent the potential usefulness of this unique cell for exploring numerous problems related to the biology of IFs and IF-MT interactions.

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