# AN ANALYSIS OF COLLAGEN SECRETION BY ESTABLISHED MOUSE FIBROBLAST LINES

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ABSTRACT

*In vitro* synthesis of collagen by established mouse fibroblast lines has been examined by electron microscopy. During rapid growth (log phase), when collagen could not be detected in the cultures, the cells lacked a well developed granular ergastoplasm and Golgi system. Upon cessation of growth (stationary phase), collagen accumulated in the cultures and the cells demonstrated highly developed granular and smooth ergastoplasm. Collagen appeared to be synthesized in the rough-surfaced endoplasmic reticulum and to be transported as a soluble protein to the cell surface by vesicular elements of the agranular ergastoplasm. Fusion of the limiting membranes of these vesicles with the cell membrane permitted the discharge of the soluble collagen into the extracellular space, where fibrils of two diameter distributions formed. The secretion of collagen is concluded to be of the merocrine type. Alternative theories of collagen secretion are discussed and the data for established lines compared with the results of other *in vitro* and *in vivo* studies of collagen fibrillogenesis.

## INTRODUCTION

The recent development of established mouse lines capable of *in vitro* collagen synthesis (1) has provided another opportunity to study the biogenesis of this molecule. This system offers the following advantages: (*a*) the cell lines may be propagated indefinitely; (*b*) the cell lines have been carried for approximately 100 generations in culture under constant conditions and are relatively homogeneous; (*c*) replicate cultures of a given line may be employed to correlate chemical and ultrastructural data with defined phases of growth; and (*d*) the cells, cultured in Petri dishes, spread out in a plane parallel to that surface. This allows defined orientation of thin sections with respect to this plane of the cell layer, and resolution of problems of geometric interpretation regarding postulated sites of fibrillogenesis.

The present report defines the range of *in vitro* fibroblast ultrastructure from the initial period of rapid division to the later stage of non-proliferation. Stages of cytodifferentiation are correlated with the appearance of extracellular collagen fibrils and the accumulation of hydroxyproline in the cultures.

A dualistic theory of collagen biosynthesis would hold that fibrils of collagen are first assembled in the ectoplasm of the fibroblast and then shed into the extracellular space, where they grow by the secondary deposition of monomeric units from solution. An alternative theory would be that the fibroblast secretes only soluble monomeric units which aggregate extracellularly to form identifiable fibrils. Both theories have been set forth in earlier studies (2–13). The present study of estab-

lished lines provides ultrastructural evidence that the aggregation of collagen fibrils is purely an extracellular event and indicates the manner in which the soluble units of collagen are secreted.

## MATERIALS AND METHODS

### *Cell Lines and Culture Conditions*

The conditions of establishment and *in vitro* growth of these mouse fibroblast lines have been reported in detail elsewhere (14). 3T6 and 3T12A are two such spontaneously evolved lines shown to produce collagen. P-3T3-1A is a line converted to collagen production by polyoma virus infection of a non-collagen synthesizing established mouse line (15). All cell lines were grown in plastic Petri dishes with the Dulbecco and Vogt modification of Eagle's medium (16) supplemented with 10 per cent calf serum. All inoculations and transfers were as trypsinized cell suspensions.

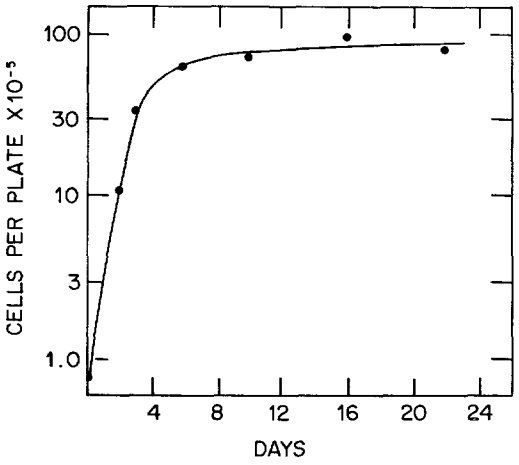



FIGURE 1 Growth of cell line 3T6. The semilog plot was derived from cell counts of trypsinized, replicate cultures. Day 0 was 24 hours after inoculation. A confluent monolayer was formed when approximately $10^6$ cells were present. Thereafter, continued growth produced multiple cell layers flattened in the horizontal plane.

### *Electron Microscopy*

Replicate cultures were washed with phosphate-buffered saline (PBS) (pH 7.2) at 37°C and fixed in one per cent $OsO_4$ in PBS at 0°C. Fixation, dehydration and embedding in Epon were performed in the original plastic culture plates as previously reported (1). Cultural morphology was excellently preserved throughout these procedures. After embedding, the cultures were examined by phase microscopy and multiple representative areas selected for sectioning. The embedded cell layer was easily cleaved from the underlying culture plate by the application of a mild shearing force. Blocks were removed by saw from the embedded material and remounted to give sections parallel or perpendicular to the horizontal plane of the cell layer. Thin sections were floated onto 200-mesh copper grids without substrate support, and stained with freshly prepared 2 per cent uranyl acetate. The latter gave better results than solutions allowed to stand for any period of time.

A correlative study of ultrastructure with all stages of growth was made for lines 3T6 and 3T12A. The results were similar in the two cases. Only stationary phase cultures of line P-3T3-1A were examined. They did not differ qualitatively from stationary phase cultures of the other two lines. Unless otherwise indicated, all electron micrographs are of line 3T6.

TABLE I

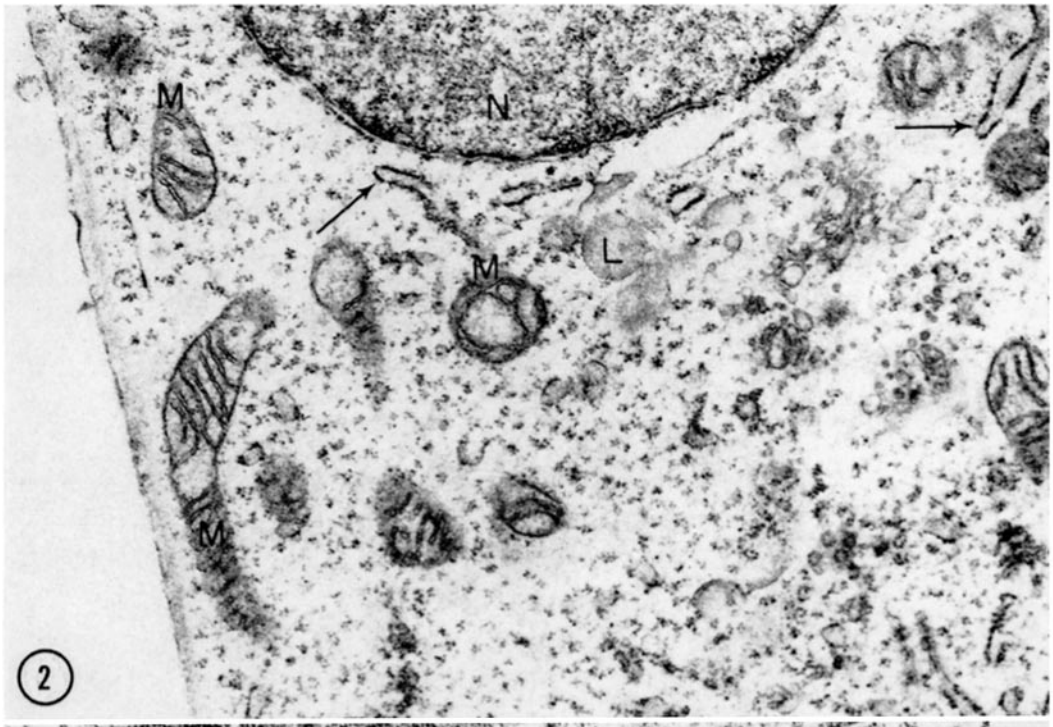
*Hydroxyproline Concentration in Cell Layers After Different Times in Culture Without Transfer*

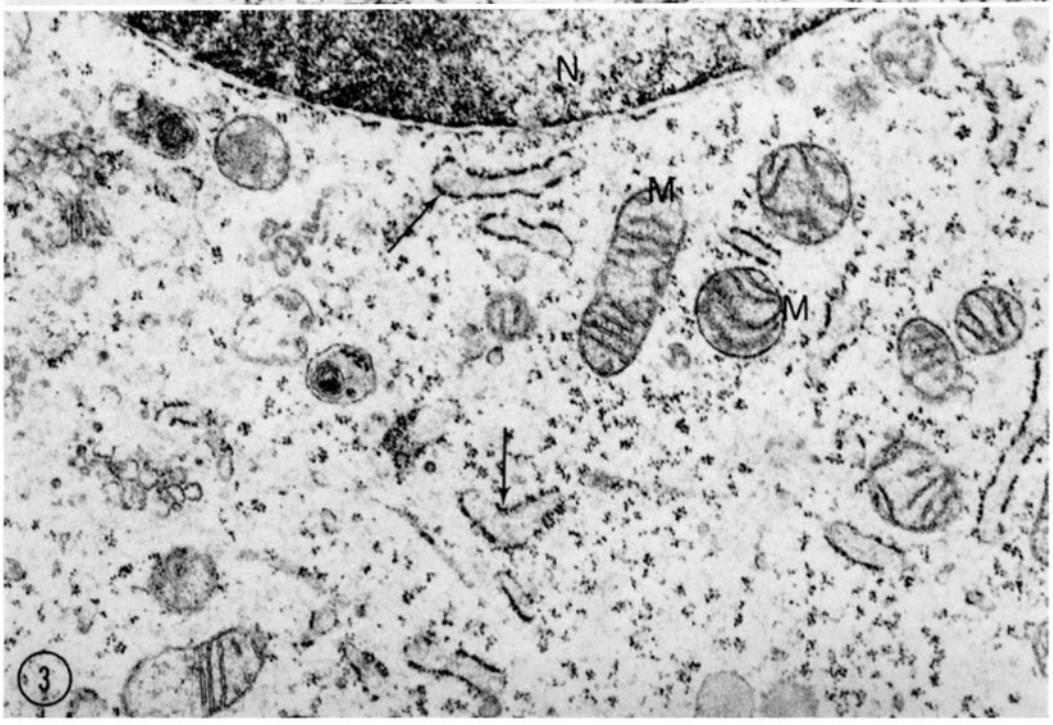
| Day | Hydroxyproline in cell layer millimicromoles per $10^7$ cells |
|---|---|
| 0–4 | None detected |
| 5 | 4 |
| 6 | 24 |
| 10 | 92 |
| 16 | 163 |
| 22 | 264 |
| 24 | 288 |

### *Hydroxyproline Determinations*

Medium was removed from the culture, and the cell layer washed twice with PBS at 37°C. The cell layer was then scraped into a tube and hydrolyzed for 15 hours in 6 N HCl at 120°C. The hydrolysates were analyzed for hydroxyproline by the method of Prockop and Udenfriend (17). Non-specific color formation was found to amount to 17 millimicromoles

FIGURES 2 and 3 Log phase. The rough-surfaced endoplasmic reticulum (arrows) is rudimentary and contains material of low contrast. Clusters of ribosomes are scattered throughout the cytoplasmic matrix. Mitochondrial profiles (*M*) are cylindroid or spheroid. The cell surface is at left in Fig. 2. *L* indicates lipid; *N*, nucleus. Fig. 2, × 21,000; Fig. 3, × 22,000.


M
N
M
L
M
2


N
M
M
3

per $10^7$ cells (18). Values obtained in the experiments reported here are corrected by this amount.

## RESULTS

A trypsinized suspension containing $5 \times 10^4$ cells[1] of line 3T6 was inoculated into each of a series of 50 mm plastic Petri dishes. The cells attached and within 12 hours began to divide. Cell counts on replicate plates, beginning the day after inoculation (day 0, Fig. 1), showed that exponential growth (log phase) continued until about day 3, with a doubling time of approximately 13 hours; thereafter, the growth rate declined and by day 6 the cells entered a stationary phase with a saturation density of about 10 million cells. The medium was changed three times weekly and the cells maintained a healthy appearance for the duration of the experiment (44 days) with little evidence of cell detachment. At day 22, a replicate culture was trypsinized and known numbers of cells inoculated onto a layer of x-irradiated feeder cells (19). Eighty per cent of the cells inoculated gave rise to macroscopic colonies, demonstrating that cells in saturation density cultures were substantially all viable.

### *Hydroxyproline Content of Cultures*

Collagen synthesis was followed by hydroxyproline determinations of replicate cultures at different times. A comparison of Table I and Fig. 1 shows that the cell layers during log phase did not contain hydroxyproline; nor were significant quantities of this molecule secreted into the media during this interval (18). On day 5, collagen synthesis began and continued at a constant rate for at least 24 days of this experiment. Therefore, the ultrastructural elements of log phase cells should not reflect the synthesis, accumulation, or secretion of collagen molecules, but only the level of differentiation of cells undergoing rapid division. In contrast, the ultrastructure of stationary phase cells should reflect collagen synthesis if every cell

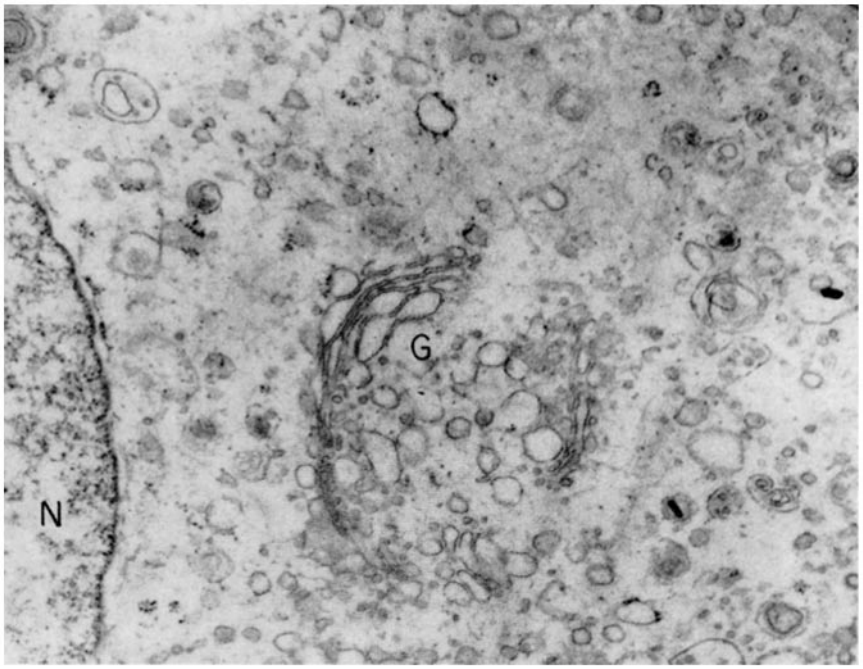



FIGURE 4 Log phase. The vesicles and flattened cisternae of the Golgi zone (*G*) are few in number and generally appear empty. *N*, nucleus. × 26,500.

[1] Previously carried through two transfers during which the cells were not permitted to reach saturation density.

in the culture is able to synthesize and secrete this molecule.

### *Homogeneity of the Cultures*

The controlled conditions under which established lines 3T6 and 3T12A were developed (constant inoculation density and constant transfer interval through approximately 100 generations) would tend to select a homogeneous cell population. Thus, given established lines developed in this manner may be distinguished by their growth rates, saturation densities, sensitivity to contact inhibition, and capacity for collagen synthesis (14, 1, 18). To test the homogeneity of line 3T6 with respect to collagen synthesis, cloning experiments were performed. A 22 day culture was trypsinized and Petri dishes inoculated with very dilute cell suspensions, so that not more than two or three widely separated colonies grew out per plate. These colonies were isolated in cloning cylinders by the method of Puck *et al.* (19), transferred with trypsin, and grown out into mass cultures. No larger than average colonies were selected, to avoid spurious clones actually arising from cell clumps. Seven clones were isolated, and five of these were successfully grown to saturation density. Hydroxyproline determinations indicated that all five clones produced quantities of collagen in the range of 5 to 16 $\mu g/10^7$ cells per day. It therefore seems very probable that every cell of the original uncloned 3T6 line used in the ultrastructural studies to be described was able to synthesize collagen.

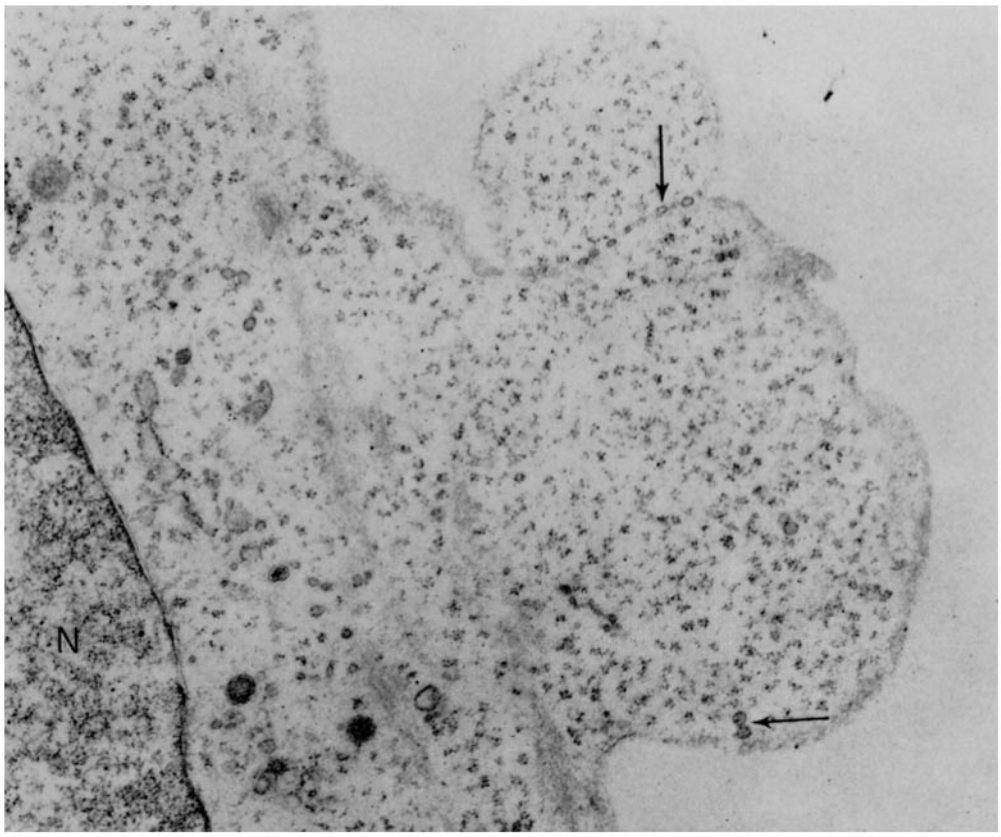



FIGURE 5 Log phase. Clusters of ribosomes and a few small vesicles (arrows) are the major elements at the cytoplasmic periphery. The cell membrane is not well defined (see text). *N*, nucleus. × 19,000.

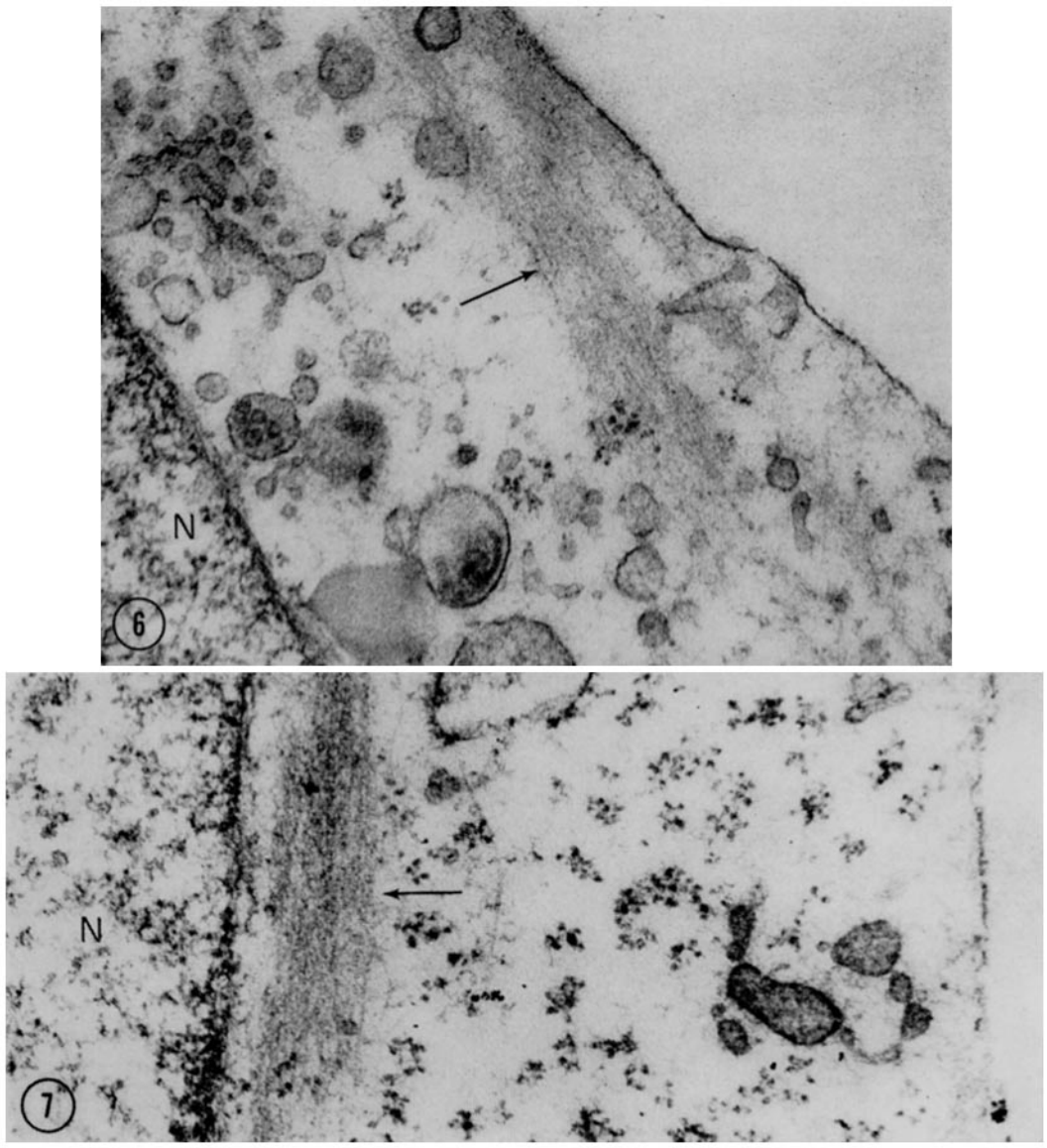



FIGURE 6 Log phase. Delicate fibrils (arrow) are present just beneath the cell membrane. Vesicular elements with limiting membranes of varying density are present at all levels within the cytoplasm. *N*, nucleus. × 55,000.

FIGURE 7 Log phase. The cell surface is at the right, the nucleus (*N*) to the left. Cytoplasmic fibrils (arrow) are aggregated adjacent to the nucleus. × 64,000.

### *Ultrastructure of Cells During Log Phase Growth*

Replicate cultures of 3T6 were examined on days 0, 1, and 2, and the following is a description of mitotic interphase cells during that interval.

The flattened, stellate-shaped cells were characterized by a poorly developed endoplasmic reticulum and a large complement of free ribosomes. When encountered, the rough-surfaced endoplasmic reticulum consisted of isolated, small

profiles containing material of low density (Figs. 2, 3). Similarly, the Golgi zone of membranes was minimally developed, with its vesicular component generally appearing empty (Fig. 4). Small vesicles similar to those seen in the Golgi zone were occasionally identified near the plasma membrane. The free ribosomes were frequently arranged in clusters (Figs. 2, 3, 5, 8), as would be expected for cells engaged in protein synthesis (20). These clusters often were the predominant particulate element at the cytoplasmic periphery. The sectioned mitochondria generally appeared small and spherical, displaying simple cristal arrangements and matrices of low density (Figs. 2, 3). Mitochondria and rudimentary elements of endoplasmic reticulum tended to be restricted to the perinuclear zone. Lipid was occasionally present in log phase cells. The cell membrane was well defined when cut at right angles (see discussion below). The cell surface occasionally demonstrated villiform or bulbous projections (Fig. 5), presumably reflecting surface movements. Surface invaginations were noted and vesicles, with limiting membranes of a density similar to that of the cell membrane, were seen in profile deep in the cytoplasmic matrix.

Delicate fibrils, approximately 50 A in diameter, could be found scattered throughout the cytoplasmic matrix; occasionally, however, they occurred in large numbers in roughly parallel array. The latter arrangement was most often seen just beneath the plasma membrane (Fig. 6), but occasionally occurred adjacent to the nucleus (Fig. 7) or elsewhere in the cytoplasm (Fig. 8). In view of the hydroxyproline data, these fibrils are not considered to be aggregates of collagen molecules. The intercellular space contained some cellular

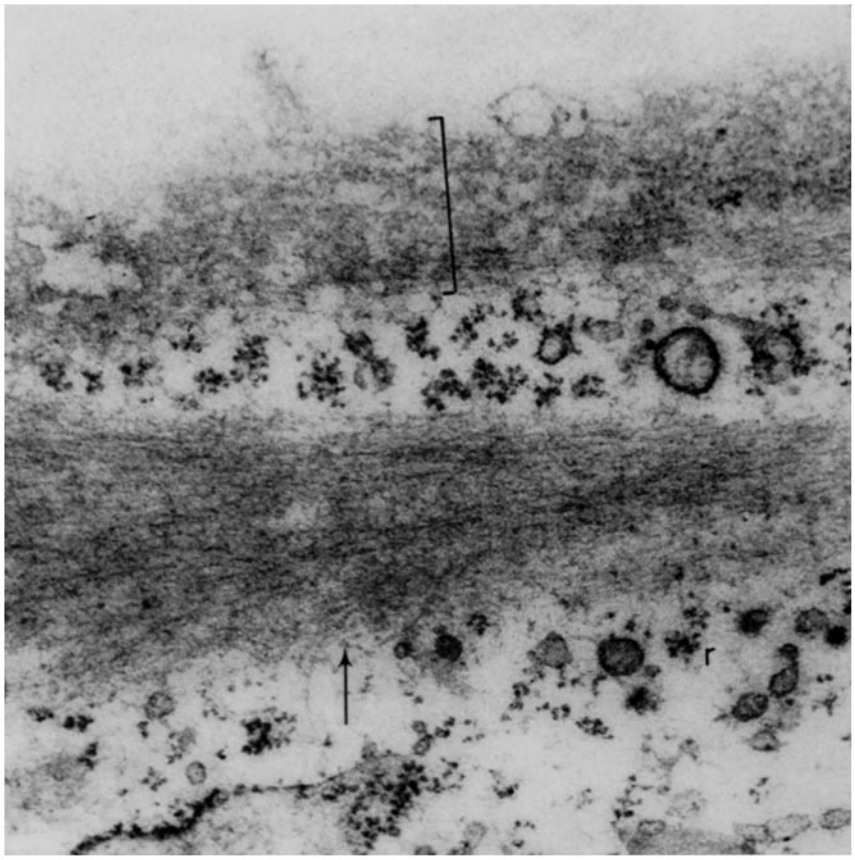



FIGURE 8 Log phase. Bracket indicates cell surface (see text). Cytoplasmic fibrils (arrow) are aggregated some distance from that surface. Ribosomal clusters (*r*) are evident. × 55,000.

debris, but no extracellular fibrils were identified in log phase cultures.

### *Ultrastructure of Cells During Phase of Diminishing Growth Rate*

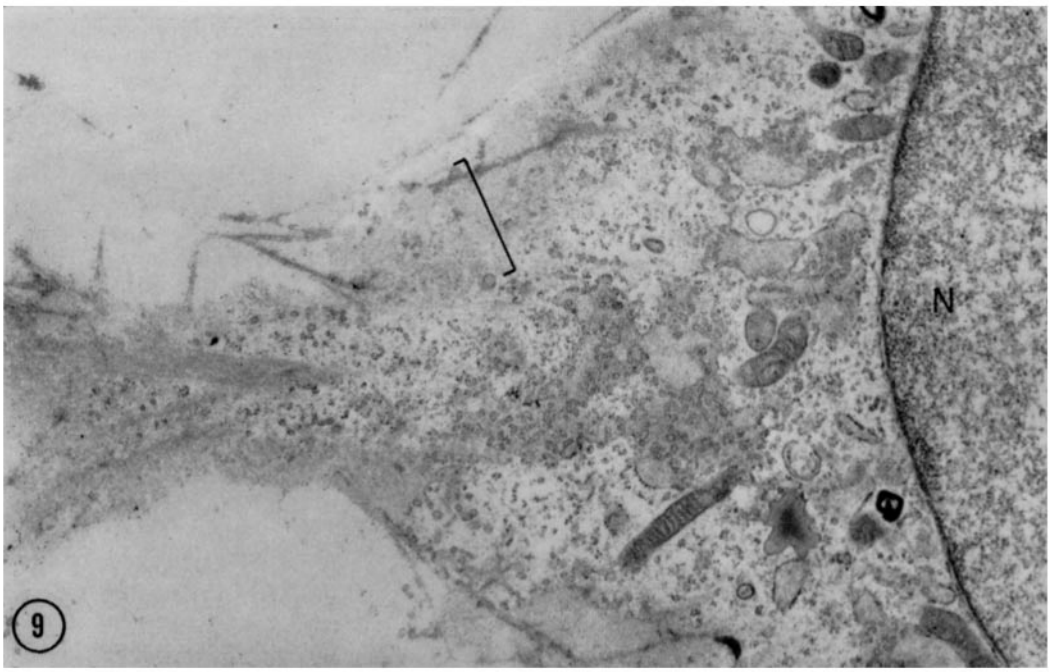
Sampling of cells on day 4 revealed some increase in number and size of profiles of rough surfaced endoplasmic reticulum. Cisternal contents were also of increased density. Mitochondria appeared more elongate with matrices of greater density. The cells did not otherwise differ in detail from those observed during log phase. Small accumulations of extracellular fibrils measuring between 50 and 100 A, though very sparse, were now occasionally identified in the intercellular spaces. Their presence suggests that a few cells had already entered the stationary phase of growth by day 4.

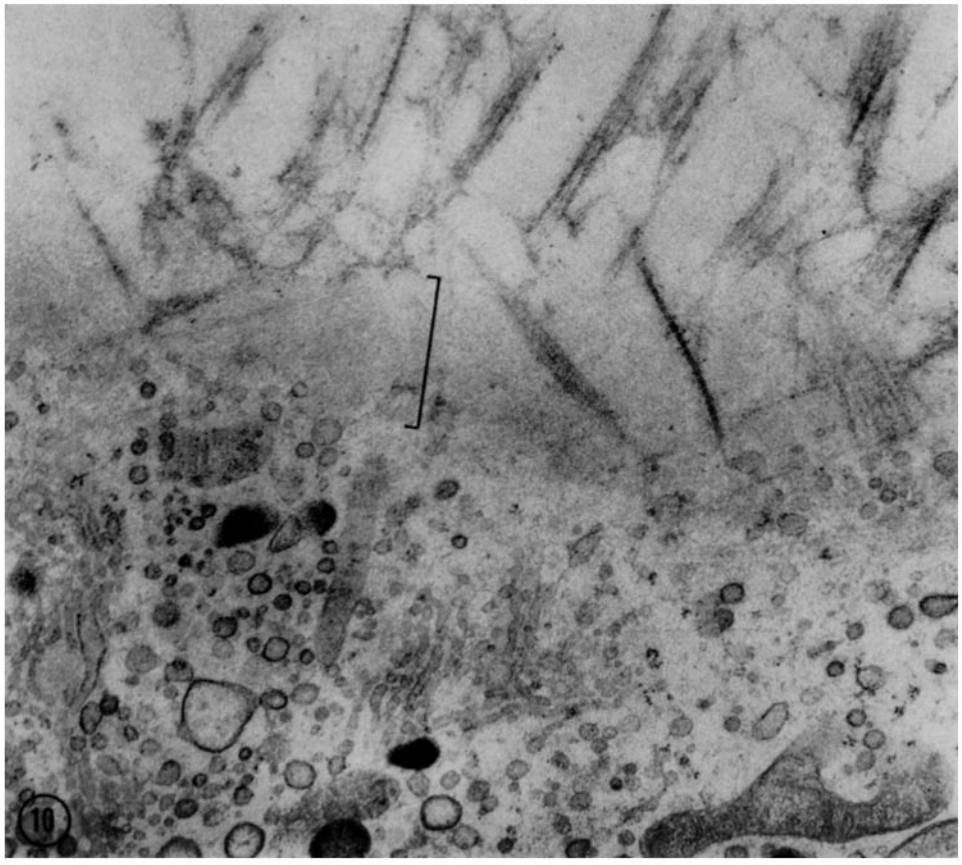
### *Ultrastructure of Cells During Stationary Phase*

Cultures were examined on days 7, 9, 13, 20, 27, and 44. The cultures were now many cell layers thick. Thus, vertical sections could contain overlapping cytoplasmic extensions from as many as 20 to 30 cells. In sections cut horizontally through the cell layers (Figs. 9 to 12) the cells appeared to lack a dense, 70 A thick cell membrane along almost their entire circumference. Instead, a widened zone of relatively lower density was present at the periphery, the ectoplasm appeared to be shedding into the extracellular space, and fibrils with and without periodicity appeared to be forming locally. Based on exactly this type of morphologic evidence *in vitro* (5, 6) and *in vivo* (3, 7, 8), it has been suggested that intracellular fibrils aggregating in the ectoplasm of fibroblasts are released by disintegration of the overlying cell membrane, such fibrils then forming the nuclei for growth of extracellular collagen fibers. However, when sections were taken vertical to plane of the same cell layer (Figs. 13 to 15) the cells could always be demonstrated to have well defined 70 A cell membranes over almost their entire sampled circumference. The vertical sections also illustrate the flat, discoid shape and the long, thin cytoplasmic extensions of the horizontally oriented fibroblasts. It thus becomes evident that the majority of horizontal sections would intersect cell surfaces at angles between 0° and 15° from the horizontal, and would contain an uncut segment of cell membrane between the points of surface intersection (see Fig. 13). Under these circumstances the cell membrane would be seen as a relatively wide zone of low density. For an angle of surface intersection of 5° from the horizontal and a section thickness of 500 A, the zone of membrane density would measure 5700 A in width $\left(\frac{500}{\tan 5°}\right)$, as was approximately the case in Fig. 11. When the angle of surface intersection approached 0°, as in Fig. 12, the extent of the surface membrane would not be precisely defined. It is concluded, therefore, that the illustrated instances of atypical surface membrane structure in fibroblasts are due to horizontal sectioning of flattened cells and bear no relationship to collagen secretion. Similarly, the non-collagen synthesizing log phase cells show the same phenomenon in horizontal section (Figs. 5, 8).

Electron micrographs of horizontal or oblique sections must also be cautiously interpreted when topographically assigning structures to intra- or extracellular positions (see discussions by Wasserman, and Godman and Porter, references 3 and 7, respectively). Because of the depth of field of the electron microscope, extracellular elements can be projected onto the cytoplasmic plane and appear to be intracellular. Thus our interpretation of Figs. 9 to 12 is not that the fibrils are being shed from the ectoplasm, but rather that they are extracellular to an intact cell surface cut obliquely and viewed on face. Only comparative studies with sufficiently thin sections, clearly normal to cell surfaces, will permit reasonably unambiguous spatial assignments. Figs. 6 and 7 of log phase cells meet these requirements and therefore make a case for the real existence of intracellular fibrils. However, vertical sections of stationary phase cells did not indicate any increase in the number

FIGURES 9 and 10 Stationary phase, horizontal sections. A distinct peripheral cell membrane is not seen. Instead, a wide zone (bracket) of low density marks the cell periphery. Fibrils with and without periodicity are present in and adjacent to this peripheral zone (see text). *N*, nucleus. Fig. 9, × 12,500; Fig. 10, × 26,000.


N
9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of such fibrils, again supporting the view that these fibrils do not represent collagen.

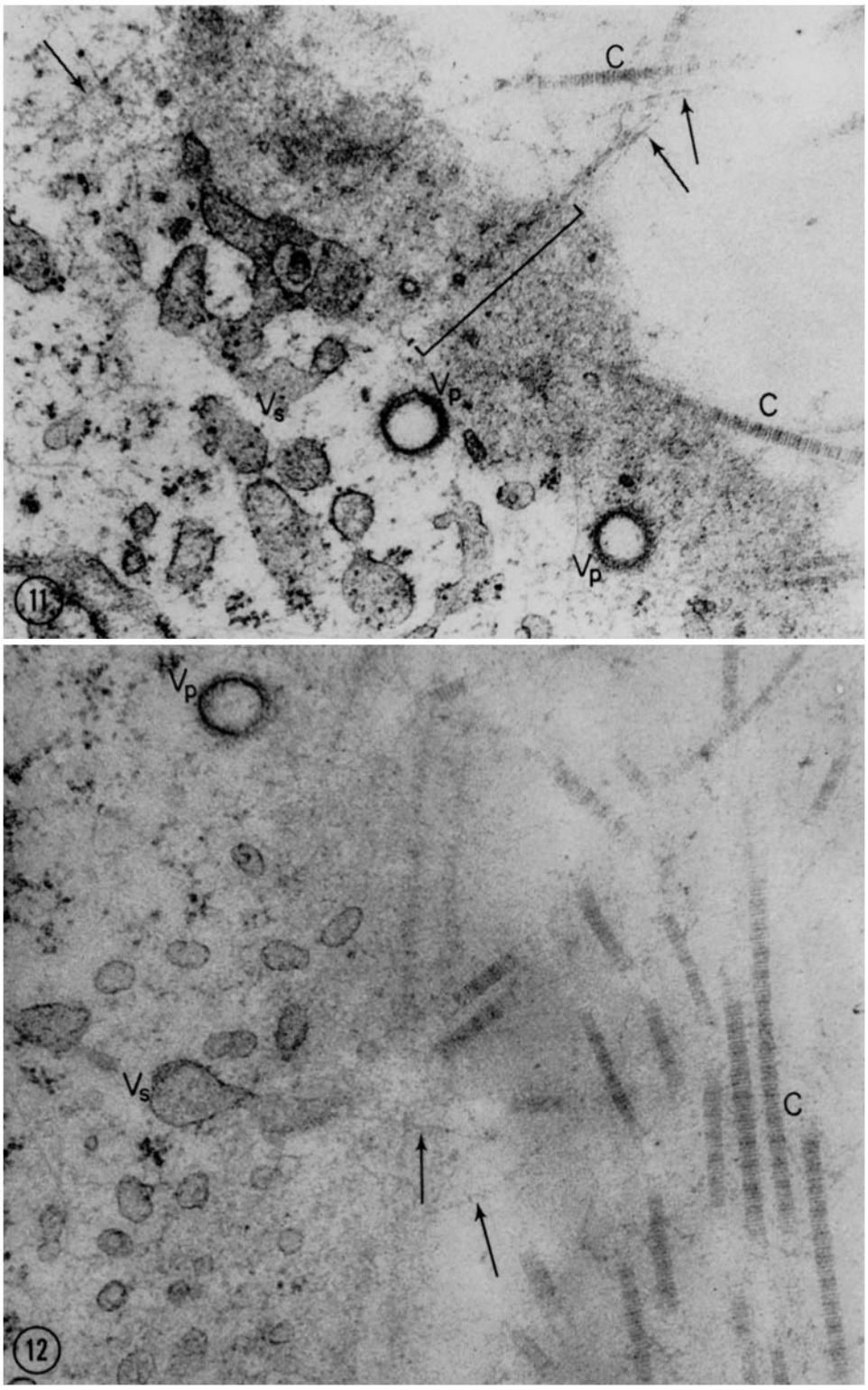
Stationary phase cells were distinguished from log phase cells by a more complex organization of mitochondria, endoplasmic reticulum and Golgi apparatus. Fig. 16 illustrates one of the remarkably elongated and branched mitochondria. The initial development of rough-surfaced endoplasmic reticulum noted at day 4 now appeared to be fully expressed. The number of such elements was increased, their continuity was apparent, and the cisternae were distended with large amounts of material of moderate density (Fig. 17). At high resolution, with heavy uranyl staining, this contained material appeared finely granular. Defined fibrils with the length, thickness and contrast of those present in the cytoplasm or the extracellular spaces were never resolved in the cisternae. The ribosomes attached to the ergastoplasm frequently formed curved chains of 10 to 12 particles (Figs. 17, 20, 21). Free ribosomes were somewhat less frequently observed than in log phase cells; they were seen mainly at the cell periphery and again occurred in clusters. The granular ergastoplasm and the mitochondria tended to be restricted to the perinuclear zone, presumably because of the extreme flattening of the cells toward the periphery.

The Golgi apparatus and associated elements of smooth surfaced endoplasmic reticulum were now also highly complex systems. The Golgi zone was often seen to be adjacent to the nucleus and to extend projections in horizontal and vertical planes between elements of rough-surfaced endoplasmic reticulum (Figs. 18 and 19). The Golgi zone contained multiple paired membranes defining narrow cisternae, and large clusters of circular, tubular, and saccular profiles. These profiles were limited by a 70 A smooth membrane of relatively low contrast. Examples were found (Figs. 20 and 21) where similar smooth-surfaced elements were in continuity with rough-surfaced endoplasmic reticulum. In such zones the smooth profiles often contained material with the same density as the contents of the adjacent rough-surfaced elements. Vertical and horizontal sections demonstrated many such clusters of agranular reticulum and associated vesicular profiles outside of the Golgi zone, particularly at or near the cell surface (Fig. 22). At the cell membrane these vesicles (designated $V_s$) contrasted in appearance with another vesicular element ($V_p$, Figs. 11, 12, 22, 24 to 26). The latter was most often regularly spherical and bounded by a smooth, but very densely staining membrane approximately 80 A in thickness. This membrane was usually bordered on its cytoplasmic surface by a condensed layer of amorphous material approximately 140 A thick. These vesicles were usually quite clear in their centers. They appeared to form from invaginations of the overlying cell membrane (Figs. 22 to 26). They thus differed from the other class of surface vesicle by their shape, the characteristics of their limiting membrane, their contents, and their presumed site of origin. Vesicles of similar structure, presumably pinocytotic in function, have been described in other cell types by Roth and Porter (21).

### *Mode of Cellular Secretion of Collagen*

The increasing quantities of hydroxyproline in stationary phase cultures may most reasonably be related to: (*a*) the accumulation of tropocollagen in cisternae of the rough-surfaced endoplasmic reticulum; and (*b*) the development of extracellular fibrils. Rough-surfaced elements could not be shown to discharge their contents into the cytoplasmic matrix nor did they communicate directly with the cell surface. They were continuous with smooth-surfaced membranous systems, however, and vesicular elements of the latter were often seen arrayed at the cell surface. Figs. 27 to 29 are further examples of the latter phenomenon. It is to be noted that the material within the vesicles remains amorphous, or at least not aggregated into clearly resolvable fibrils, and similar material forms a background density where distinct fibrils are forming just outside the cell membrane. It appears that soluble collagen is delivered to the cell surface in vesicular elements of the agranular ergastoplasm. Fusion of their limiting membranes with the overlying cell membrane permits the discharge of the collagen molecules from the cell.

FIGURES 11 and 12 Stationary phase, horizontal sections. Periodic (*C*) and non-periodic fibrils (arrows) are present at surfaces where no typical laminar, peripheral cell membrane can be identified. Bracket indicates cell surface in Fig. 11. Two classes of vesicles at cell surface are labelled $V_p$ and $V_s$, respectively (see text). Fig. 11, × 55,000; Fig. 12, × 65,000.


C
Vs
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11
Vp
Vs
C
12

The other class of vesicle at the cell surface ($V_p$), characterized by a different type of limiting membrane, is presumed to represent a pinocytotic rather than a secretory element.

We were unable to judge whether the secretory vesicles migrated from the Golgi zone or represented a process of ribosomal detachment and direct vesiculation of the endoplasmic reticulum at the cell periphery. There is little evidence in our system that the Golgi zone represented a site of

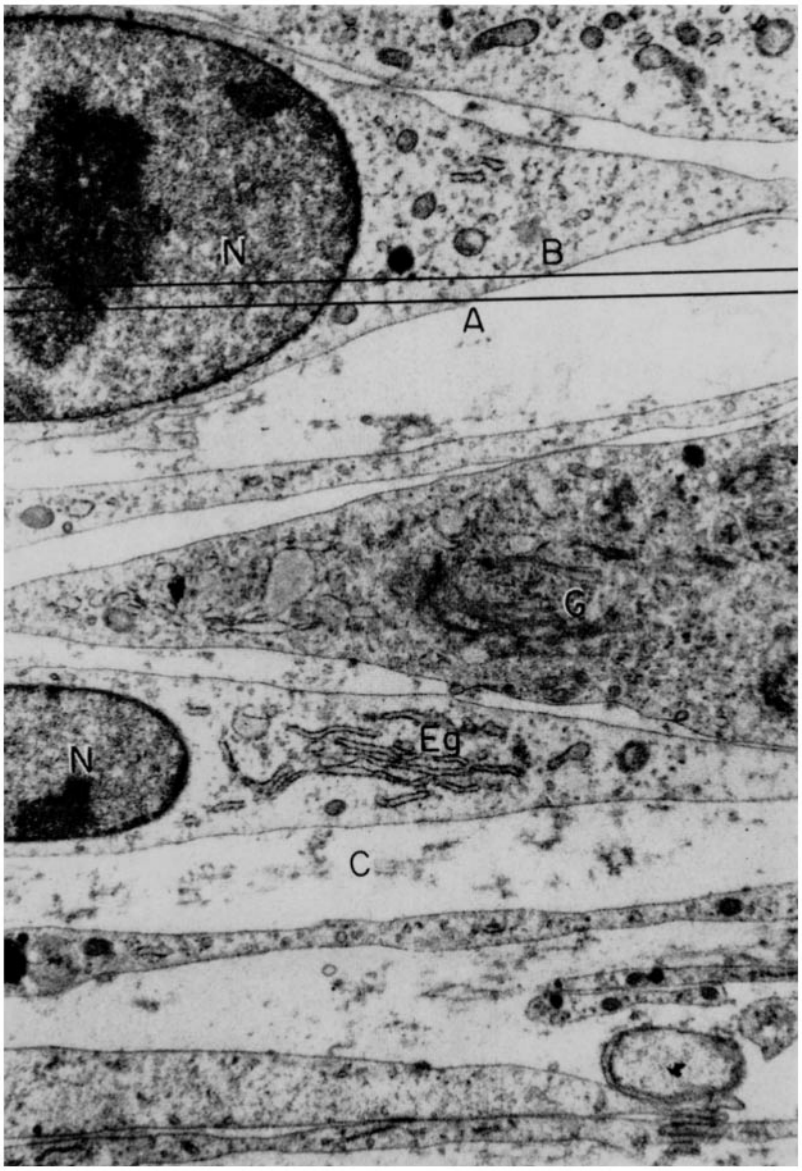



FIGURE 13 Stationary phase, vertical section. The cell membranes have been generally intersected at 90° and hence intact, dense, 70 A thick lines are seen at all cell surfaces. The two parallel lines indicate the manner in which a horizontal section would intersect a cell surface. The cell membrane would be intersected at small angles from the horizontal at points *A* and *B* and an uncut segment of membrane $\overline{(AB)}$ would be viewed on face. The result would be the visualization of the cell surface as in Figs. 9 to 11. *N*, nuclei; *Eg*, granular ergastoplasm; *G*, Golgi zone; *C*, collagen. × 9,000.

relatively static storage and concentration of large quantities of tropocollagen. It was characterized as an area containing many small vesicular and long tubular elements with contents of only moderate density, rather than large spaces filled with extremely dense material. In the few instances where the cells contained extraordinary amounts of stored material, it was always contained in the cisternae of the rough-surfaced endoplasmic reticulum (Fig. 30). Therefore, the Golgi apparatus in these cells appeared particularly adapted to the rapid and continuous transfer of synthetic products and/or as a source of membranous elements for the reticulum network.

### *Extracellular Fibrillogenesis*

Once the majority of cells reached stationary phase (day 5), hydroxyproline synthesis began at a

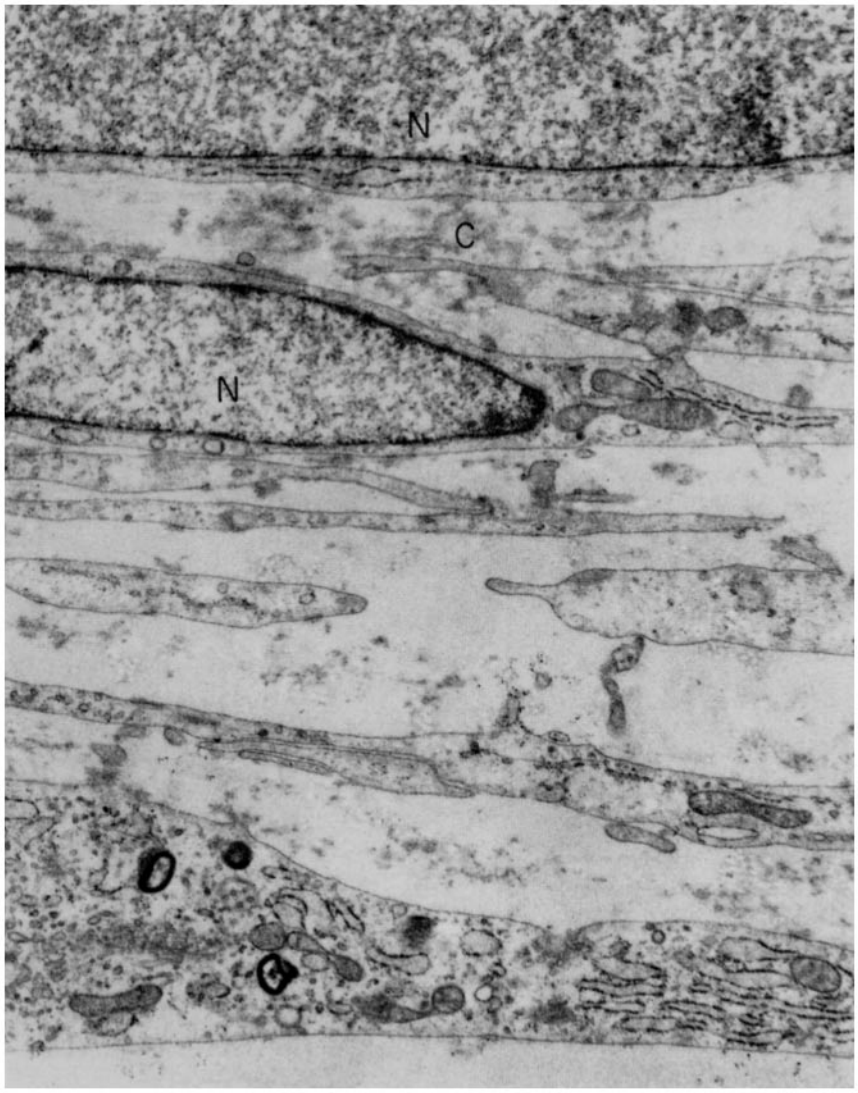



FIGURE 14 Stationary phase, vertical section. The cell membranes are seen as intact, dense, 70 A thick lines. The lower margin of the photograph is parallel to the horizontal plane of the original culture plate. The flattening of the fibroblasts is evident. Collagen (*C*) appears as amorphous intercellular material at this magnification. *N*, nuclei. × 12,500.

maximal rate and large numbers of non-periodic extracellular fibrils appeared. These fibrils ranged from 40 to 160 A in diameter (Fig. 31) and some as small as 70 A in diameter displayed a primitive beading along their length. Between day 7 and day 10 thicker fibrils identifiable as collagen appeared (Figs. 31 to 33). These fibrils typically possessed a periodicity of about 550 A and the asymmetric intraperiod banding pattern of native collagen. However, in the thinnest of these fibrils (160 to 250 A) the measurement of the macroperiod could become uncertain, as the individual bands sometimes appeared of equal intensity and thickness at the magnifications employed. With longer times in culture the proportion of periodic to non-periodic fibrils increased. Non-periodic fibrils could be found, however, at all times, particularly near the cell surfaces. Individual fibrils were generally of uniform diameter with lengths too great to be measured in a given section. Fibril diameters were measured in cultures of varying age and the data are presented in Table II and Fig. 34. Table II demonstrates that no more than two classes of fibrils were present throughout the experiment, non-periodic fibrils of approximately 85 A diameter, and periodic fibrils of about 400 A diameter. The mean fibril size in each category did not change significantly through the 44 days of the experiment, although the proportion of fibrils belonging to the periodic class rose with time. The frequencies of occurrence of individual fibril diameters irrespective of time in culture are plotted in Fig. 34. It is evident that there is virtually no overlapping of the two distributions.

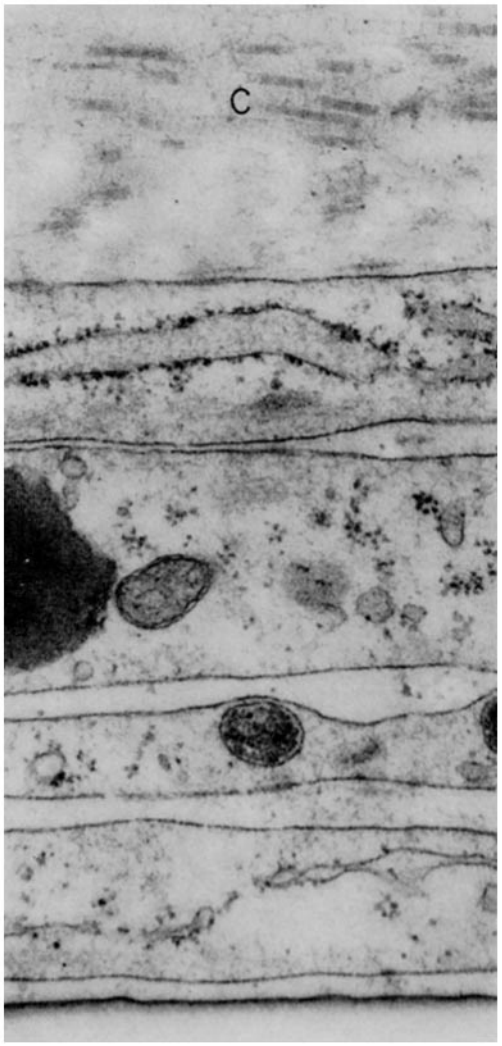



FIGURE 15 Stationary phase, vertical section. The cell membranes are well defined. The bottom black line indicates the plane of cleavage from the plastic culture plate. The precise parallel orientation of the cells is evident. A horizontal section through such an area could give a view of the cell surface as in Fig. 12. *C*, collagen fibrils in extracellular space. × 44,000.

The correspondence between fibril appearance and onset of hydroxyproline synthesis suggests that secreted soluble collagen units quickly form extracellularly into long fibrils of the 85 A diameter range. Once such fibrils begin to grow again by deposition of monomers along their length, they very rapidly achieve diameters of about 400 A. Therefore, further increase in their cross-sectional areas must occur very slowly. The increasing amounts of hydroxyproline in these cultures must therefore depend upon a continuous increase in fibril number (and perhaps length) rather than continued growth in diameter beyond the 400 A range of a relatively fixed number of fibrils.

### *Additional Observations*

The collagen fibrils were not uniformly distributed throughout the intercellular spaces nor did they occupy a large fraction of that volume even at day 44. The long axes of the fibrils were oriented parallel to the horizontal plane of the cell layer. Small sets of contiguous fibrils had their long axes parallel over segments of their length, but these mutually parallel segments could assume any angular distribution in the horizontal plane.

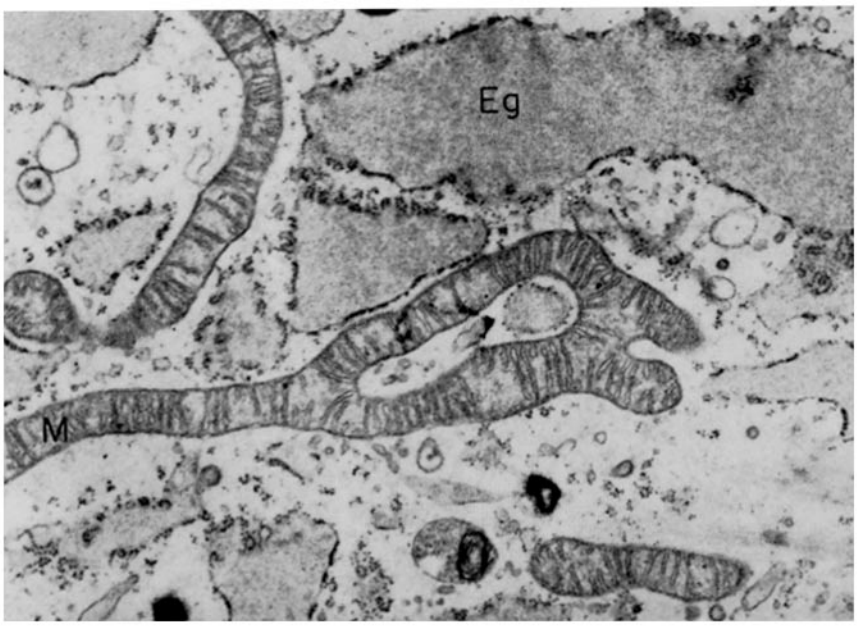



FIGURE 16 Greatly elongated, bifid mitochondrion (*M*) in a stationary phase cell. Compare with mitochondria in log phase cells (Figs. 2, 3). $E_g$, granular ergastoplasm. × 19,000.

Thus vertical sections often demonstrated fibrillar sets each defined by their angle to the plane of sectioning (Figs. 35 and 36). Small groups of fibrils could often be found in declivities or grooves in the fibroblast surface (Fig. 37). These, as well as the variety and number of cytoplasmic projections in different planes, attested to the active surface movements of the fibroblasts. The cells appeared to be capable of phagocytosis, for fibrils, cellular debris, and foreign particles from the medium could be identified at the cell surfaces (Fig. 38), within surface invaginations and in cytoplasmic vacuoles. Late stationary phase cells demonstrated increasing numbers of membrane-limited cytoplasmic bodies of varying size, containing material of varied structure (Figs. 39 to 41). The latter included dense granules, vesicular profiles, and complexes of membranes, often in compact lamellar configuration. These cytoplasmic bodies or granules were most often found at the periphery of the Golgi zone.

In cultures from all three cell lines an ordered array differing from that of native collagen was occasionally observed. Figs. 42 and 43 illustrate this other hierarchy of fibrils. The larger of these aggregates were fusiform in shape. The distance between repeating units averaged 850 A, the darker intraperiod band measuring about 400 A. Non-periodic fibrils of 70 A thickness or less could be resolved within the fibers.[2] No instance was found of structural continuity between this system and banded collagen fibrils. Where the two classes juxtaposed there was no tendency for their individual elements to come into register. Moreover, there is no obvious resemblance between the various demonstrated types of reconstituted collagen (22, 10) and the fibers in question. It therefore cannot be concluded that the latter necessarily belong to the collagen class of proteins. However, these fibers might represent the three-dimensional aggregation of non-periodic collagen fibrils, before monomer deposition from solution could convert each of them into a typically banded collagen fibril. It is noteworthy that the fiber of Figs. 42 and 43 is similar to periodic elements described by Jakus in the limbal portions of Descemet's membrane of the cornea (23).

## DISCUSSION

Explants or primary cultures have been employed by Porter and Vanamee (24), Jackson and Smith

[2] The terminology of Wood and Keech is used (39). Fibril denotes an element apparently undivided at the magnification used; a fiber indicates a structure resolvable into smaller units.

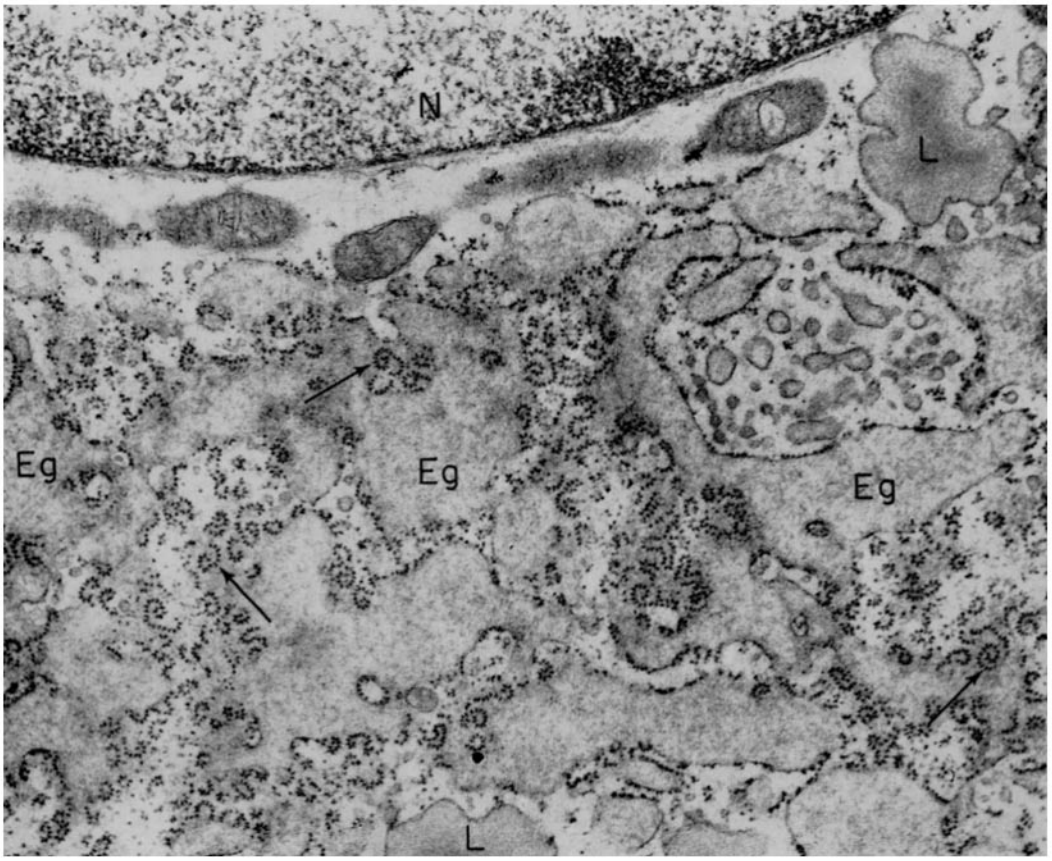
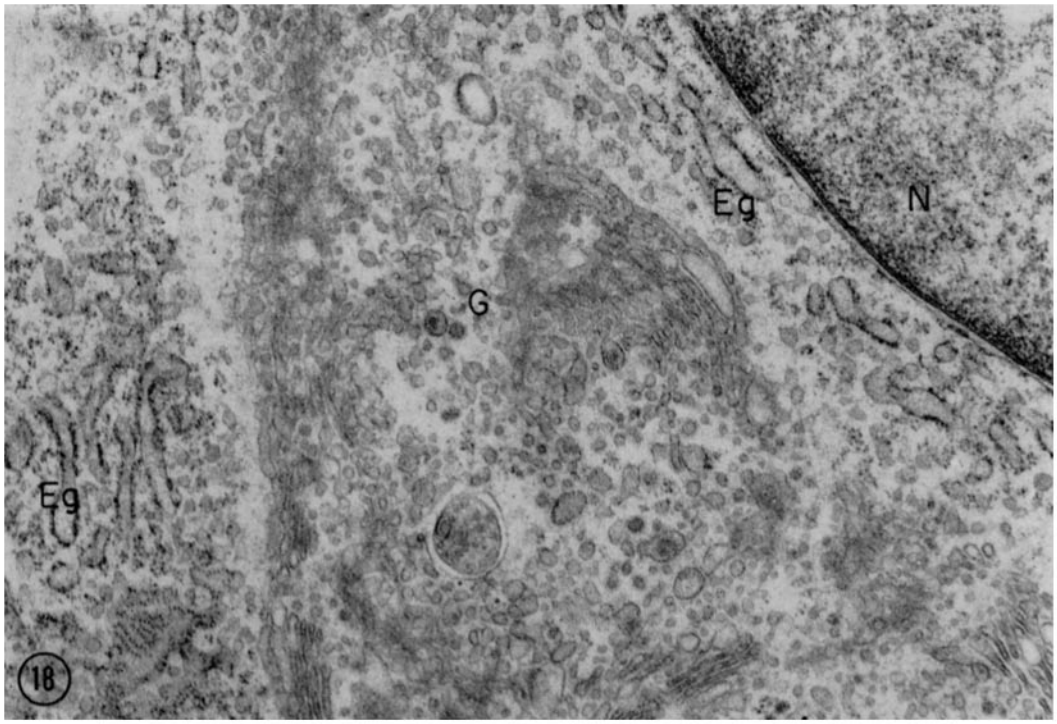


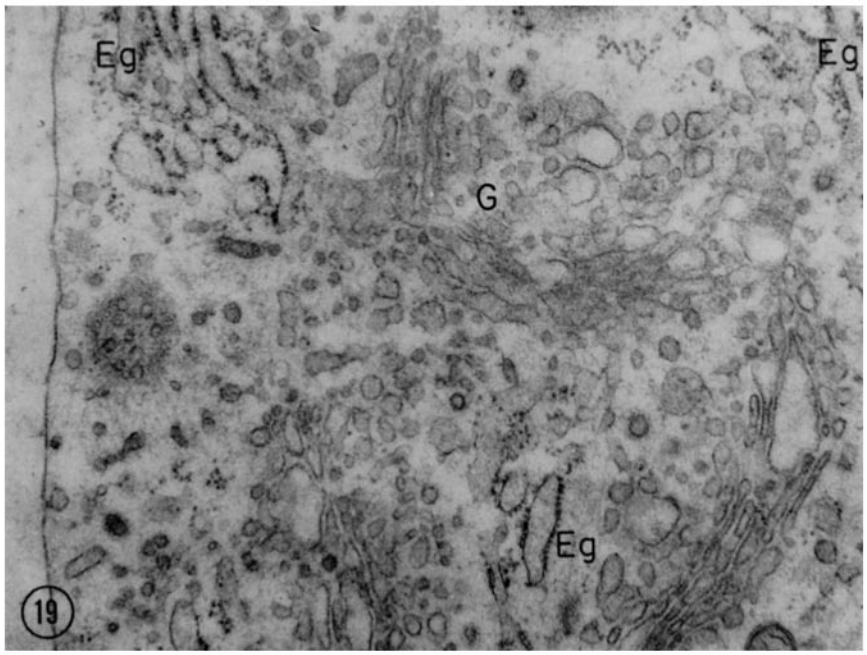

FIGURE 17 In stationary phase cells the granular ergastoplasm ($E_g$) is a large, highly branched system containing material of moderate density. The attached ribosomes tend to form short, curved chains (arrows) and they are not uniformly distributed over the ergastoplasmic surfaces. *L*, lipid; *N*, nucleus. × 25,000.

(25), Porter and Pappas (4), Yardley *et al.* (5), and Schwarz *et al.* (26), in previous ultrastructural studies of *in vitro* collagen formation. Many of our morphologic observations did not differ greatly from those in the cited works, but in certain instances the data available from established lines permitted different interpretations. There is every reason to presume that our data may be generalized to *in vivo* fibroblastic function. The ultrastructure of the established lines of fibroblasts is similar to that of fibroblasts in various adult and embryonic tissues (4, 7, 12, 13, 27, 28), granulomata (8), regenerating tendon (3, 29, 30), and healing wounds (31). The collagen fibrils produced in our cultures were structurally identical to those observed in the cited studies. Moreover, our cultures generated another class of fibrils similar to that found in the cornea, a tissue derived from mesoderm and composed in large part of collagen.

In certain respects the established fibroblast lines offer advantages for the ultrastructural study of fibrillogenesis. The homogeneity of our *in vitro* cell population ensures that all structural events observed may be assigned to the fibroblast. Cytodifferentiation may be correlated with defined intervals of rapid division and non-proliferation, respectively; as only the non-proliferating cells synthesize collagen, their ultrastructure may be interpreted in terms of this function with some confidence.

The fibroblasts dividing at a maximal rate *in vitro* resembled, in general, embryonic cell types growing *in vivo* (7, 12, 32–35). Slowing of growth rate was associated with further differentiation of




FIGURES 18 and 19 Highly developed Golgi system in stationary phase cells. Smooth-surfaced Golgi elements ($G$) are interposed between the granular ergastoplasm ($E_g$). The cell surface is at the left in Fig. 19. $N$, nucleus. Fig. 18, × 26,000; Fig. 19, × 37,000.

lamellar systems, notably the ergastoplasm and the Golgi complex. Thus, the detection of two morphologic classes of fibroblasts in studies of regenerating tendon (29) and cutaneous wound healing (31) probably represents a sampling at the two extremes of fibroblast differentiation.

We believe that the fibrils in the cytoplasmic matrix of the fibroblasts do not represent collagen precursors, for the following reasons: (*a*) there is no good relationship between the presence of such fibrils, hydroxyproline synthesis, and the appearance of extracellular collagen; (*b*) there is no morphologic evidence available relating the intracellular aggregation of these fibrils to the synthetic products in the ergastoplasm and Golgi system; (*c*) one cannot demonstrate unambiguously by electron microscopy any continuity between intra- and extracellular fibrils; and (*d*) the evidence cited for dissolution of the cell membrane as a means of releasing intracellular fibrils is better interpreted as a sectioning phenomenon. Yardley, studying horizontal and vertical sections of primary explants, concluded that extensive dissolution of the cell membrane occurred (6). However, the vertical sections in his study seem to illustrate only occasional minute ruptures in the plasma membrane that the author grants might be "preservation artifact." Moreover, he states that "perpendicularly cut thin sections . . . indicated that the commonly seen *total* absence of a visible outer cell membrane in thin sections made from the plane of growth is probably an exaggeration of the true situation . . . and no doubt results largely from acutely tangential sectioning of the cell membrane." The postulated loss of large segments of cell membrane secondary to collagen secretion would also imply that such cells would become non-viable. This clearly was not the case in our experiments, for plating efficiencies of 80 per cent were achieved with cells of a 22 day culture. Evidently, differentiated fibroblasts retained the capability for division when conditions of low cell density were restored.

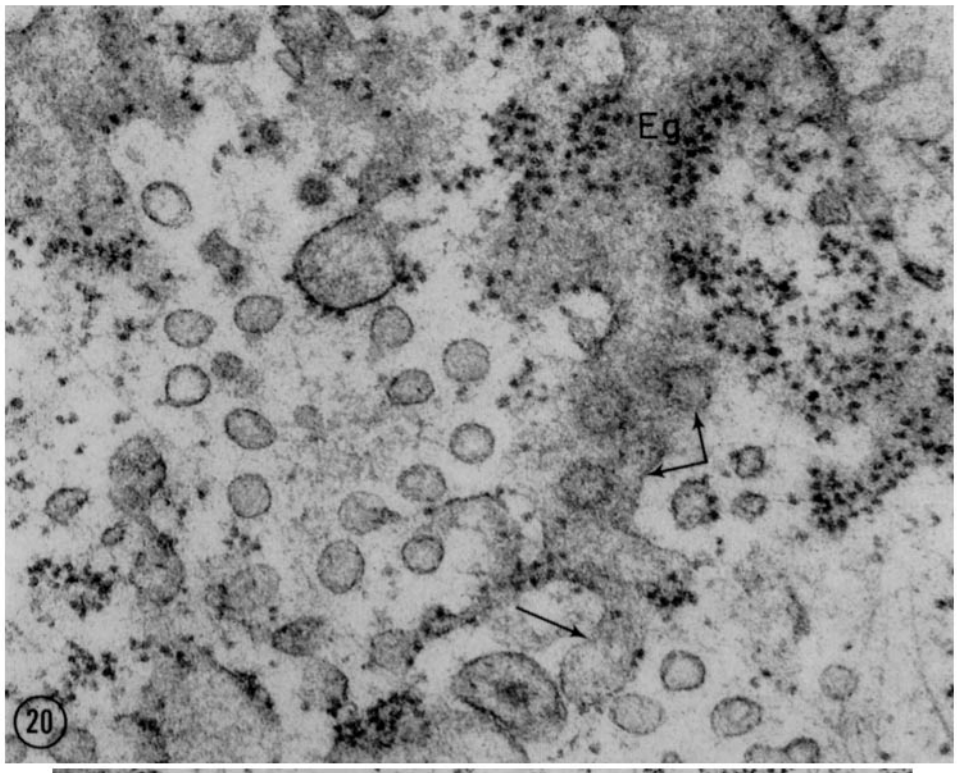
The functional significance of the cytoplasmic fibrils remains undefined. Since such fibrils seemed prominent in zones of telophase separation and pinocytotic surface activity, we suggest that they are involved in phenomena of cell motility and cytoplasmic streaming.

The depicted merocrine form of collagen secretion in our fibroblast lines does not differ greatly from a secretory mechanism suggested by Karrer (36). Siekevitz and Palade (37) and Palade (38) have shown that in the pancreatic acinar cells certain enzymes are synthesized by ribosomes attached to the reticulum network and then transported through that system to the Golgi zone for concentration and storage. Upon appropriate stimulation, such zymogen granules are released from the apical region of the cells by fusion of their limiting membranes with the overlying plasma membrane. Collagen secretion by fibroblasts therefore appears quite analogous, with the exception that there does not appear to be a prolonged phase of tropocollagen storage and concentration in the Golgi zone. Failure to demonstrate or favor the merocrine form of collagen secretion in previous ultrastructural studies might be ascribed to their dependence upon sampling of mixed cell populations inhomogeneous with respect to the function of collagen synthesis.

Granting the proposed mechanism of collagen secretion, it would seem to be necessary that the molecules not aggregate into large fibrils within the cavities of the reticulum network. Failure to do so would not appear to be a concentration effect, for the cisternae of the rough-surfaced endoplasmic reticulum appear to contain concentrations of material at least as great as are seen in the extracellular sites of fibril formation. Intracellular fibril formation could be prevented if the tropocollagen molecules were in some respect incomplete. For example, polypeptide chains could be folded to render polar side groups inaccessible for covalent or hydrogen bonding. Alternatively, the molecules could be in all respects complete but the presence of inhibitors or the rapidity of secretion could prevent intracellular aggregation. Extracellular factors could serve to remove inhibitors or to promote and complete the aggregation of the collagen molecules.

The formation of extracellular fibrils in our cul-

FIGURES 20 and 21 Stationary phase. Arrows indicate sectioned profiles of agranular ergastoplasm in continuity with granular reticulum ($E_g$). Material of moderate density is present in the cisternae of both smooth and rough elements and in the cavities of contiguous smooth-surfaced vesicles. Fig. 20, × 74,000; Fig. 21, × 50,000.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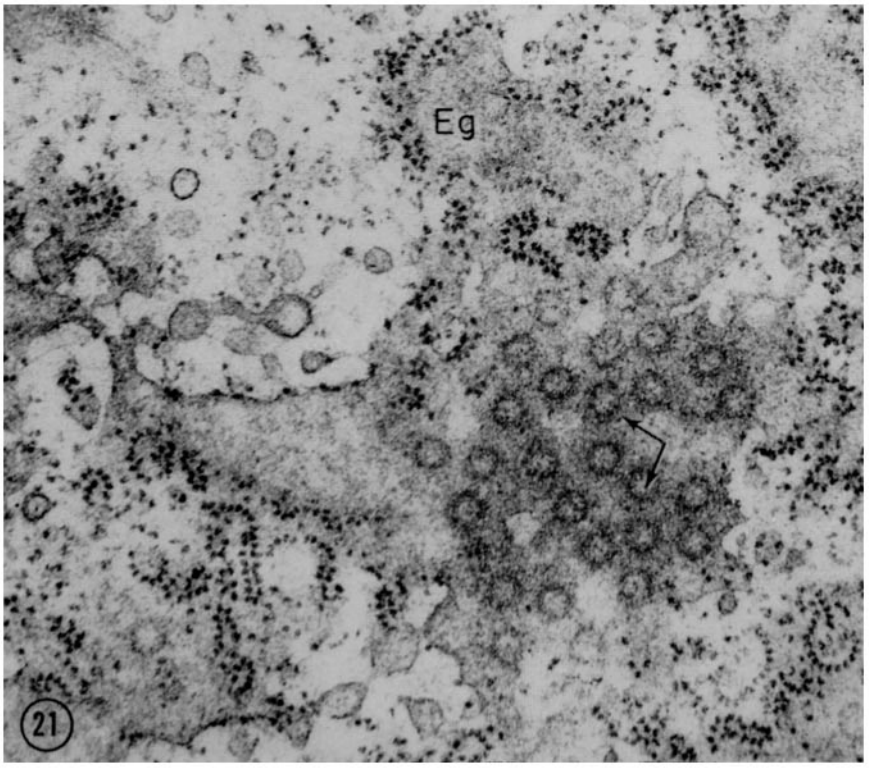
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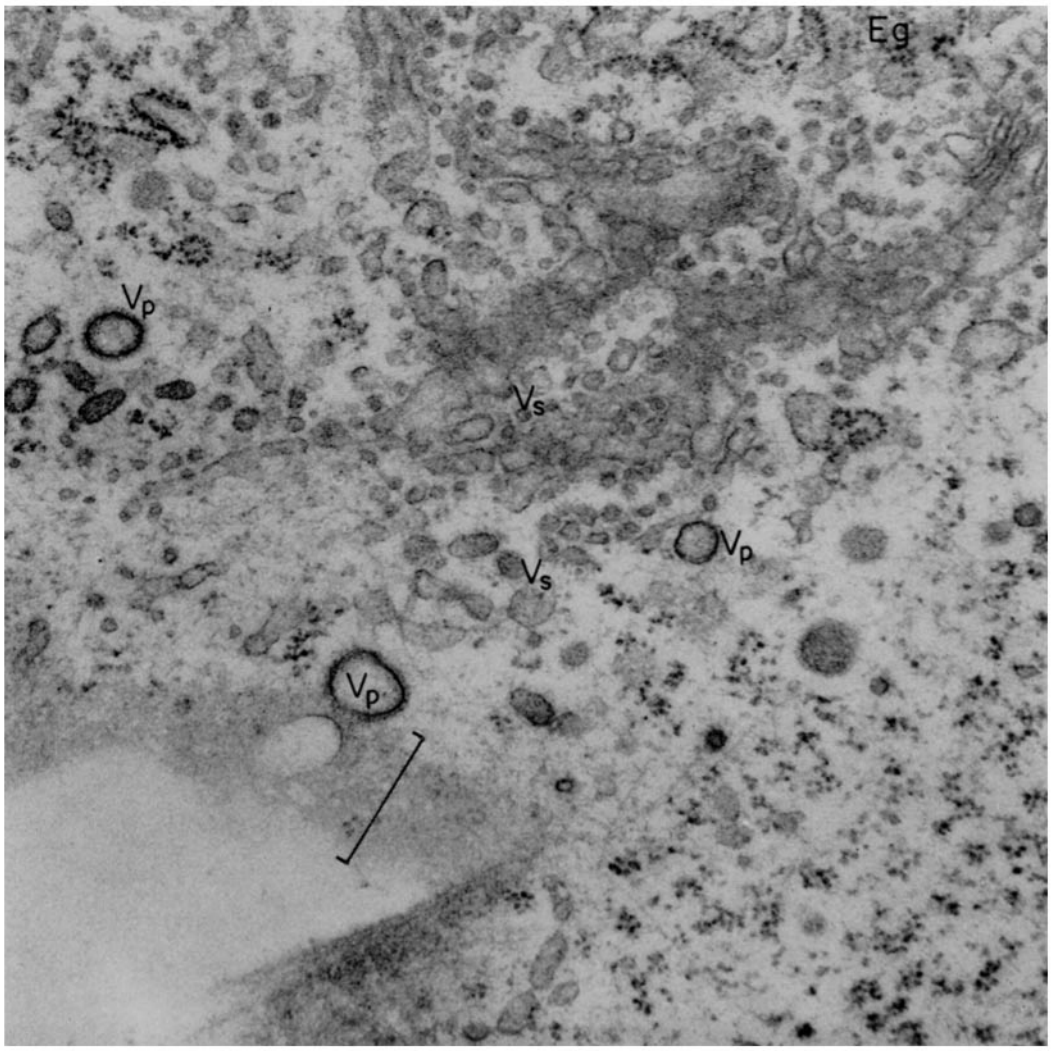
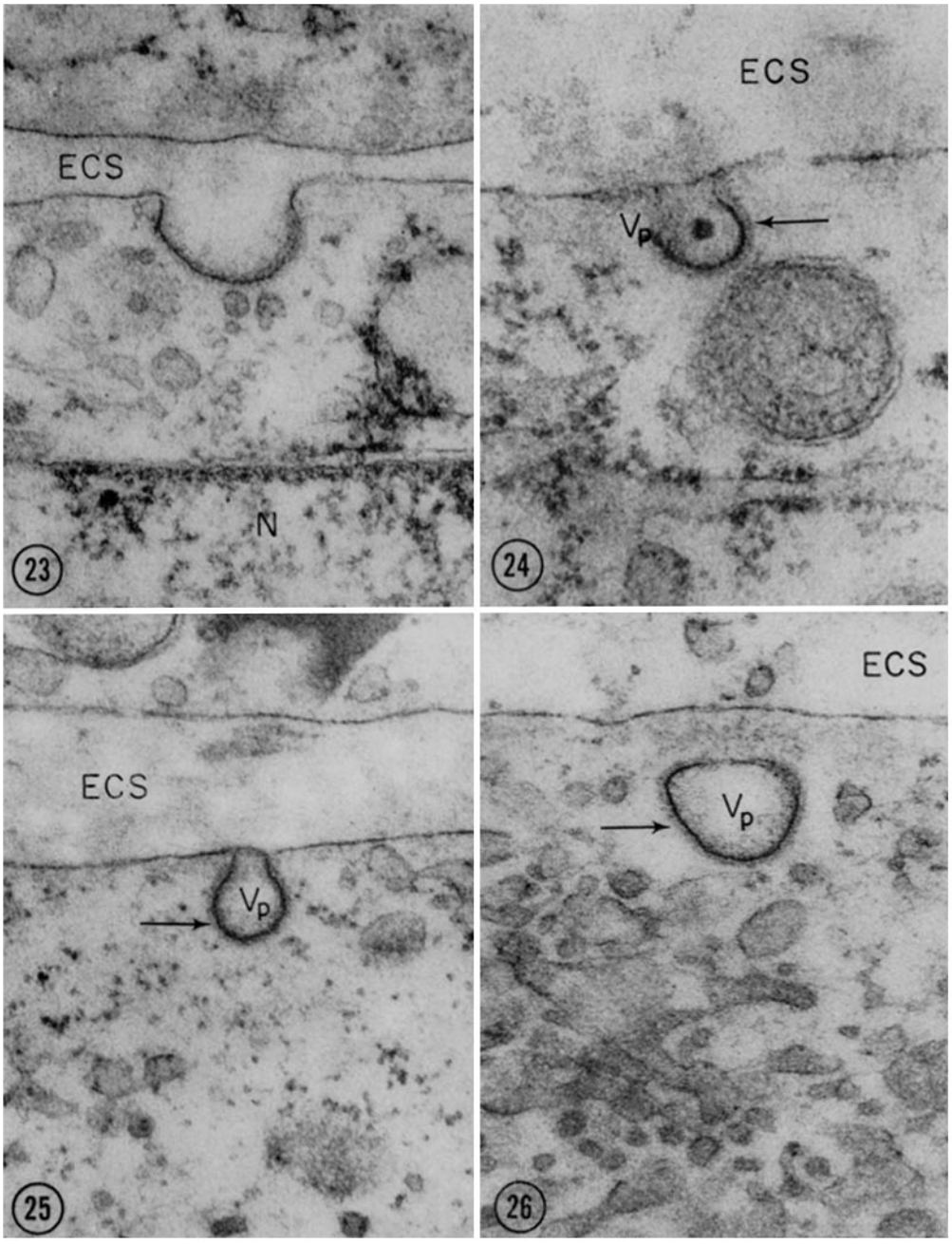


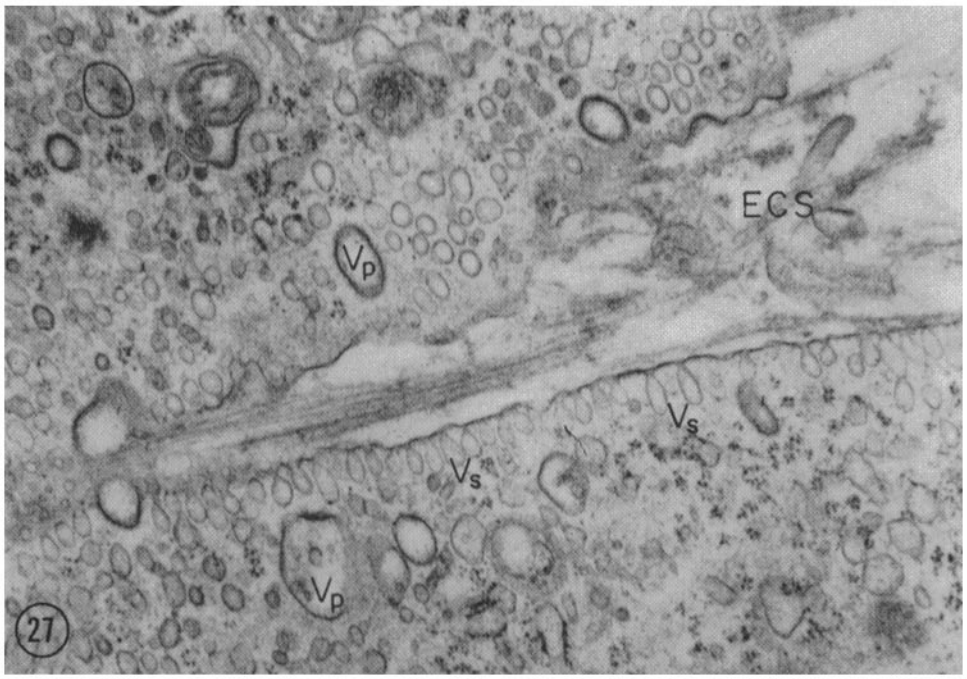

FIGURE 22 Stationary phase. Agranular ergastoplasmic elements ($V_s$) extend to the cell surface (bracket) where they contrast with another membrane-bounded vesicular element ($V_p$). $E_g$, granular ergastoplasm. × 50,000.

tures can be explained in terms of precipitation of collagen molecules from solution. Failure to find periodicity in the thinnest fibrils (< 160 A) or typical intensities of banding in somewhat larger fibrils (160 to 250 A) is explicable in terms of low density of uranyl binding and limited photographic resolution. The demonstrated modal distributions of fibril size are in accord with the studies of Wood and Keech (39) and Wood (40, 41) on the kinetics of collagen reconstitution from solution. They found that the distribution of ultimate fibril size was determined by the ambient conditions (*e.g.* temperature, pH, ionic strength) during the nucleation phase of collagen precipitation. Three *in vivo* studies (8, 30, 31) have also demonstrated distinct distributions in fibril diameters. Why fibril diameters should be limited in the presence of a continuous supply of collagen is not clear. Perhaps, once a fibril has achieved the size determined in the nucleation step, and the majority of possible intra- and intermolecular bonds have been formed, the addition of new molecules becomes very difficult on energetic grounds.

We believe the non-random geometric distribu-




FIGURES 23 to 26 Stationary phase. $V_p$ is presumed to represent a pinocytotic vesicle derived from invagination of the cell membrane (Fig. 23). As the vesicle forms (Figs. 24, 25) and migrates to the cell interior (Fig. 26), its limiting membrane acquires greater contrast and an external layer of amorphous material (arrows). A collagen fibril lies within the invagination illustrated in Fig. 24. *ECS*, extracellular space; *N*, nucleus. Fig. 23, × 74,000; Fig. 24, × 93,000; Fig. 25, × 62,000; Fig. 26, × 74,000.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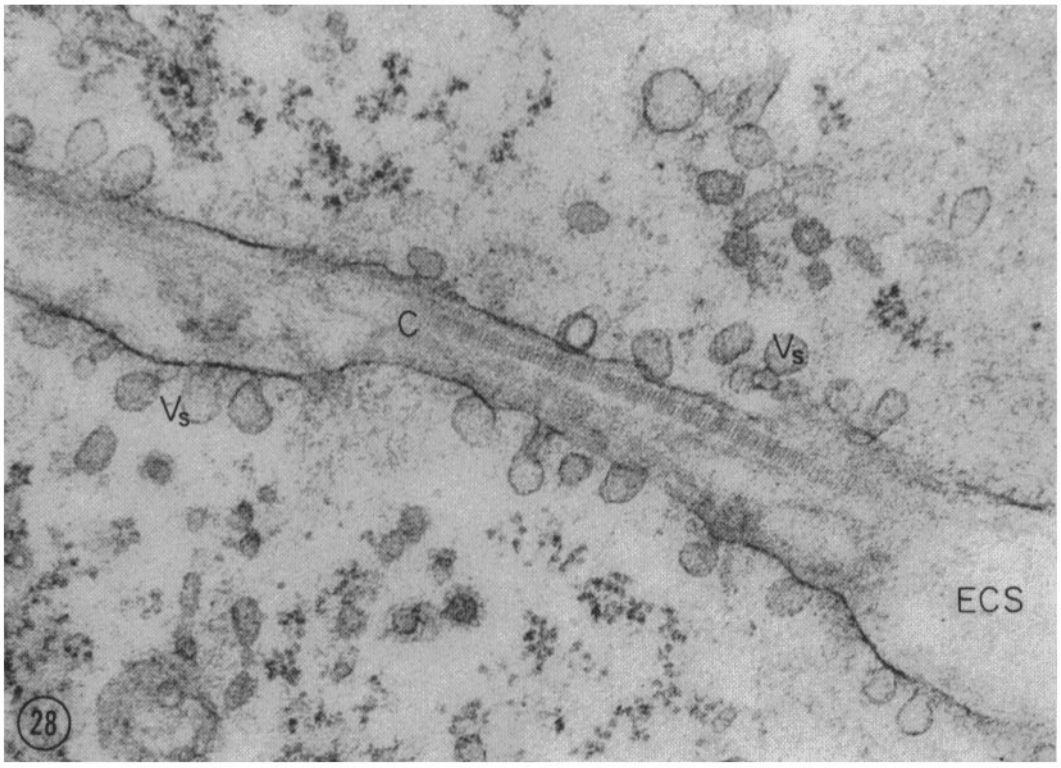
Vp
27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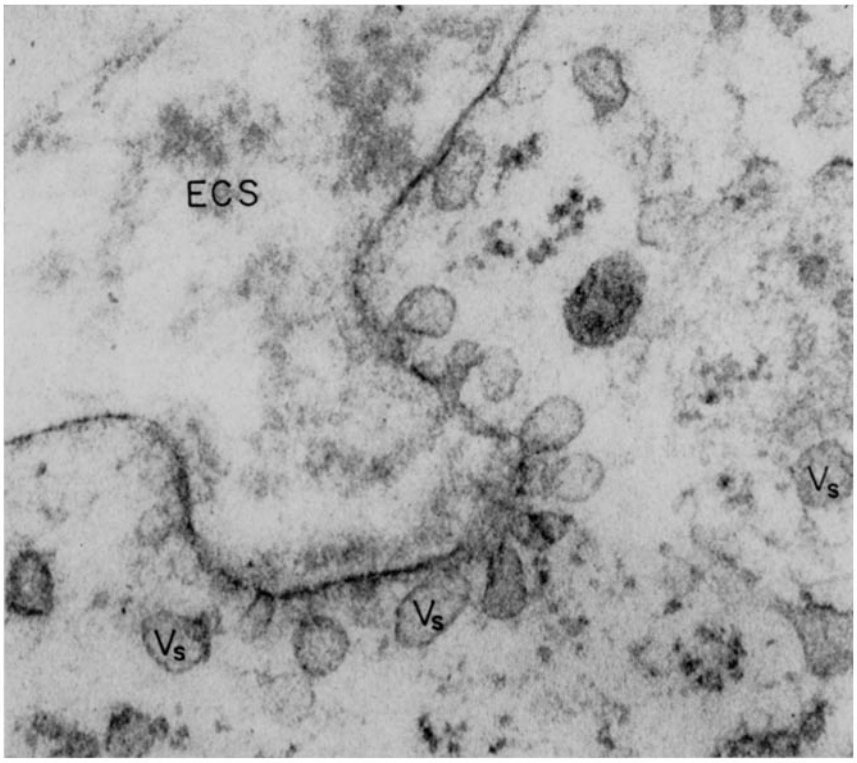


FIGURE 29 Stationary phase. Smooth ergastoplasmic elements ($V_s$) appear to discharge their contents into the extracellular space (*ECS*) after fusion with the cell membrane. × 101,000.

tion of fibrils in the extracellular space to be due to the orientation and movements of the cells. Initial fibril formation occurs upon or just adjacent to the cell surface, and often within indentations of that surface. The flattening and packing of the cells would restrict the long axes of the collagen fibrils to the horizontal plane. Cell movements in that plane would tend to entrain associated and encountered fibrillar segments, producing parallel orientation among such segments.

Examination of the vertical sections emphasizes the large volume of the extracellular space. Presumably the cells are separated by a relatively large volume of material almost completely lacking electron-scattering power. Soluble collagen may represent a fraction of this material but a significant portion must be composed of other proteins and/or mucopolysaccharides. The cell lines employed in these studies are known to produce relatively large quantities of hexuronic acid-containing mucopolysaccharides (42). The presence of a viscous solution between the closely packed cells would serve to diminish the loss of collagen molecules by diffusion. Thus, local high concentrations of collagen would be maintained, and the formation of fibrils would be favored.

*Note:* After the submission of this paper, a detailed autoradiographic and electron microscopic study of

FIGURES 27 and 28 Stationary phase. Smooth ergastoplasmic elements ($V_s$) arrayed at the cell surface fuse with the cell membrane and release their contents into the extracellular space. Non-periodic and periodic fibrils (*C*) form extracellularly. Collagen secretion is thereby judged to be of the merocrine type. $V_p$, pinocytotic vesicle; *ECS*, extracellular space. Fig. 27 (Cell line P-3T3-1A), × 32,000; Fig. 28, × 74,000.

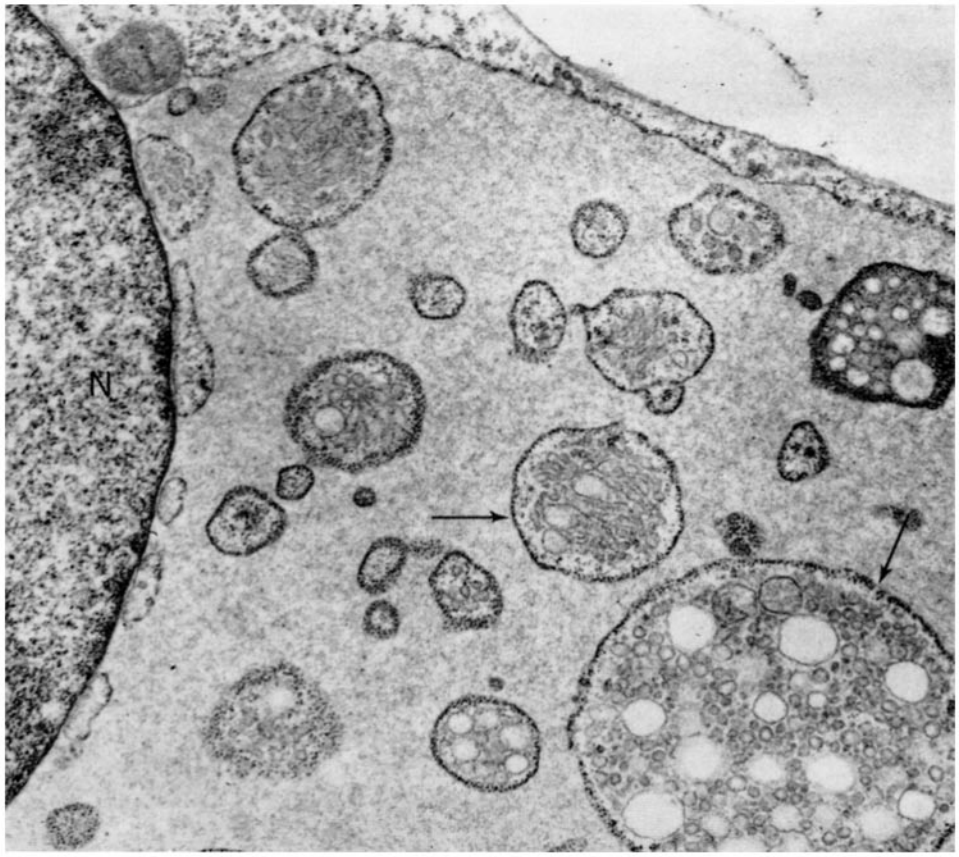


FIGURE 30. Retention of large amounts of material within the granular ergastoplasm of a fibroblast of line P-3T3-1A. Projections of cytoplasm (arrows) into the expanded cisterna enclose smooth ergastoplasm. *N*, nucleus. × 19,000.

collagen synthesis in cartilage was published by Revel and Hay (*Z. Zellforsch.* 1963, **61**, 110). This study also indicated that collagen secretion occurred by fusion of smooth vesicles with the cell surface membrane, and hence was of the merocrine type.

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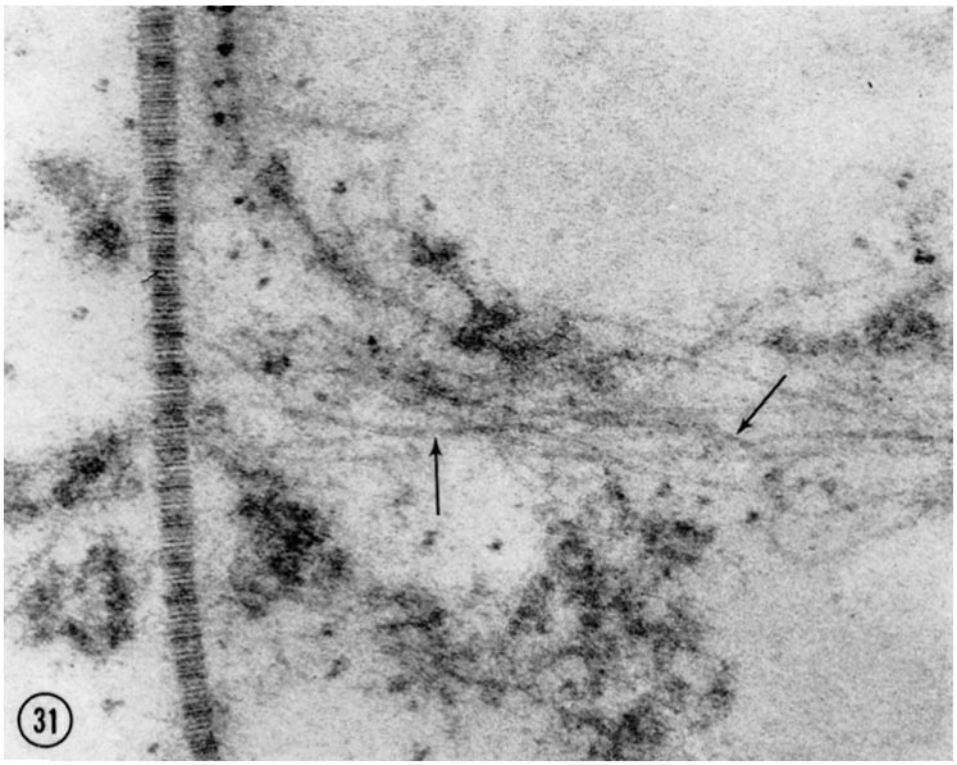
FIGURE 31 Stationary phase culture, day 9. Non-periodic fibrils (arrows) and a banded collagen fibril are illustrated. The periodic fibril measures 495 A in diameter. × 93,000.

FIGURE 32 and 33 Two fibrils of native collagen produced in these cultures. Their diameters measure 630 A and 560 A, respectively. Their period measures approximately 550 A. × 149,000.



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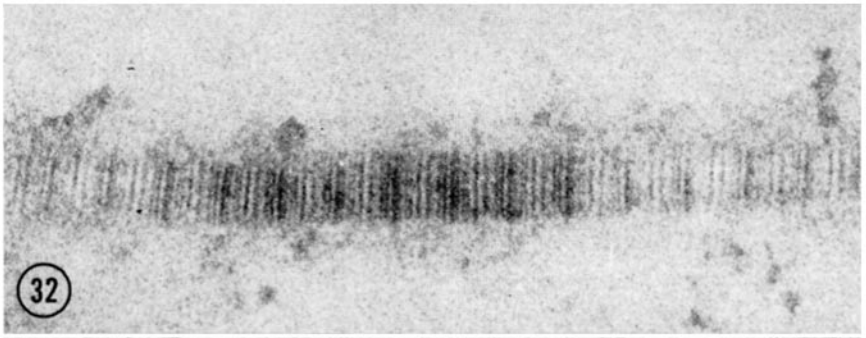

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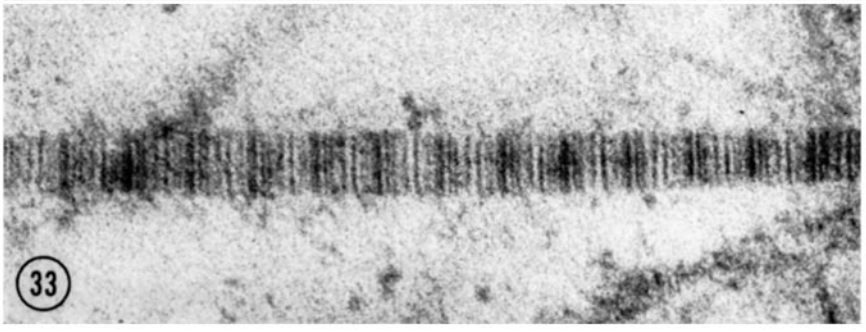

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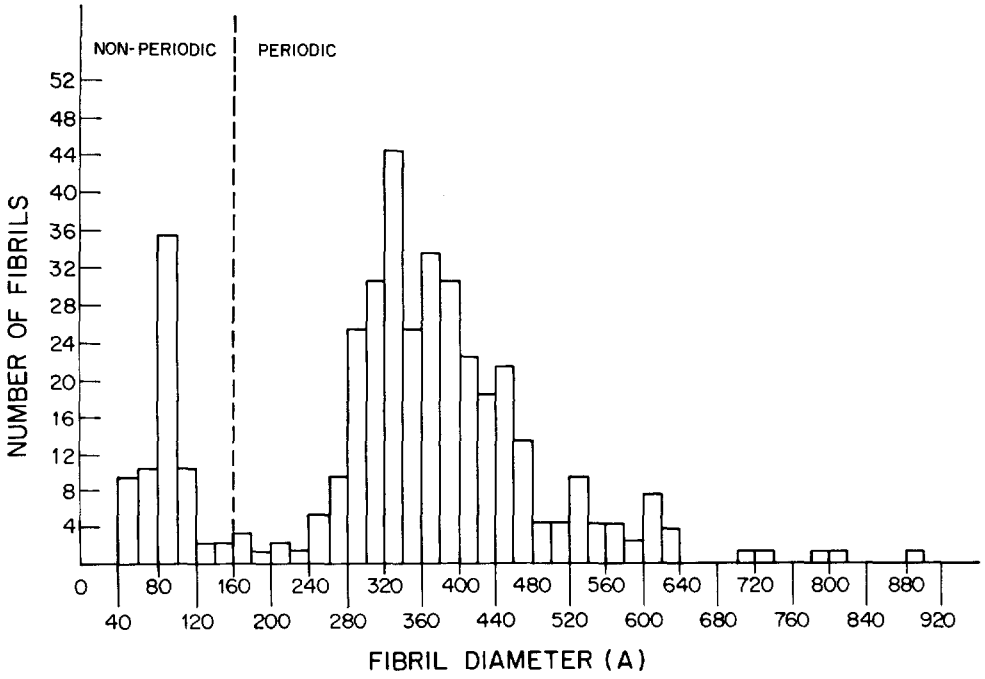


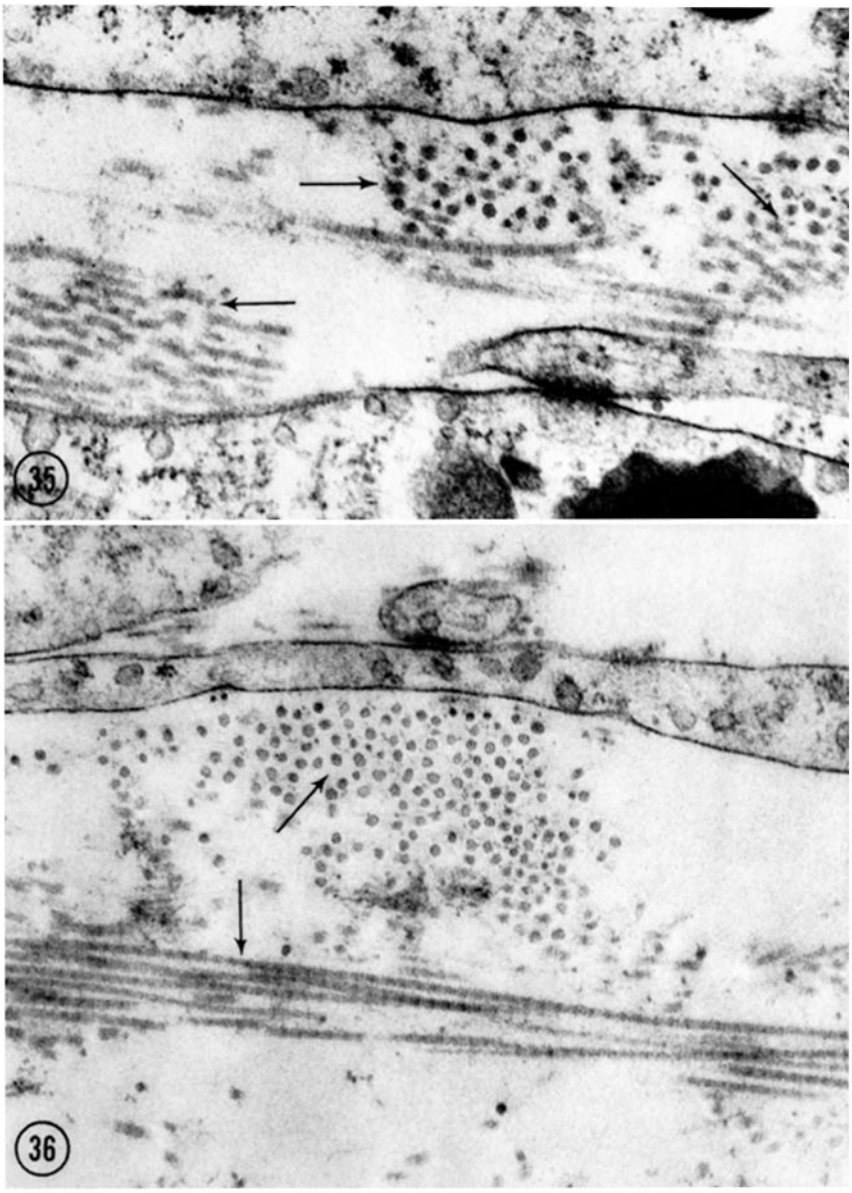

FIGURE 34 Frequency of occurrence of individual fibril diameters. At the magnifications employed, the measurements of fibril diameters below 85 A were rather inaccurate.

TABLE II

*Extracellular Fibril Diameters*

| Days in culture | Non-Periodic | | | Periodic | | |
|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n |
| | *A* | | | *A* | | |
| 7 | 83 | 31 | 14 | None present | | |
| 9 | 90 | 18 | 36 | 424 | 110 | 39 |
| 13 | 81 | 16 | 12 | 416 | 103 | 40 |
| 20 | Not measured | | | 341 | 48.9 | 107 |
| 27 | Not measured | | | 418 | 143 | 61 |
| 44 | Not measured | | | 395 | 81.2 | 78 |

Individual measurements (n) were made from prints at magnifications of 70,000 to 100,000.




FIGURES 35 and 36 Stationary phase cultures, day 44, vertical sections. The collagen fibrils occupy a small fraction of the intercellular space; their long axes lie in the horizontal plane. Sets of contiguous parallel fibrils (arrows) are defined by their angle to the vertical plane of sectioning. Fig. 35, × 50,000; Fig. 36, × 36,000.

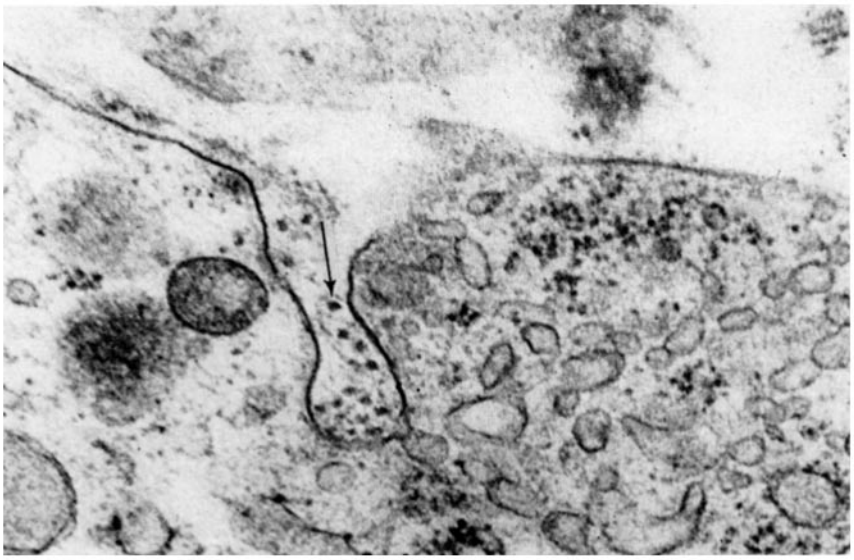


FIGURE 37 Cell line P-3T3-1A. Collagen fibrils (arrow) lie within a groove in the fibroblast surface. × 68,000.

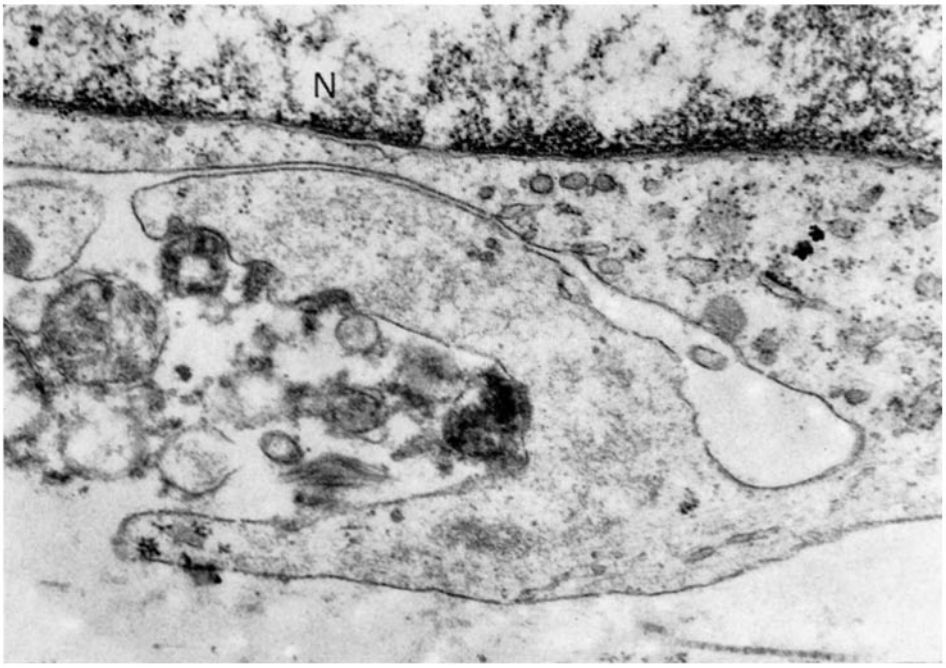
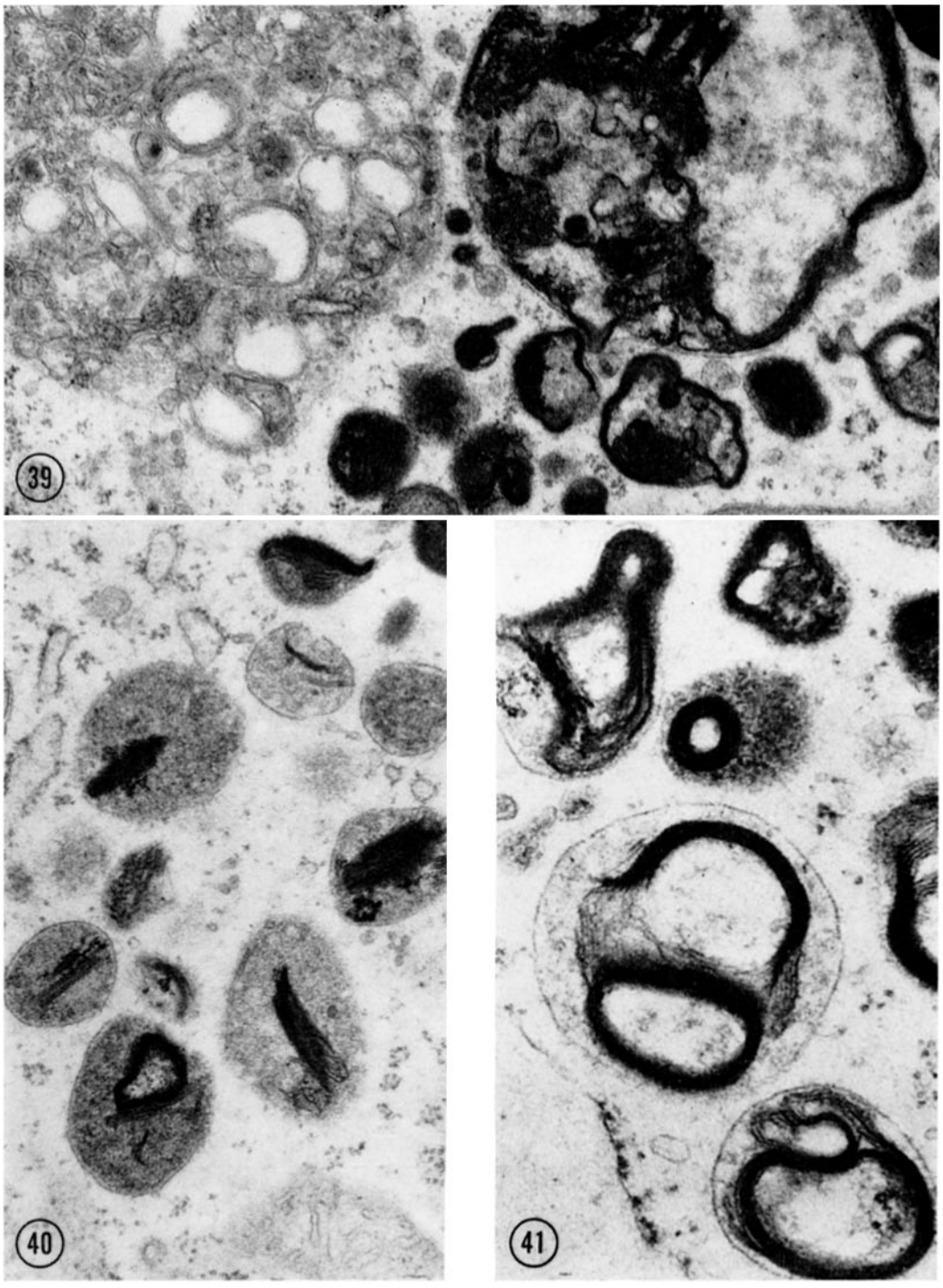



FIGURE 38 A cytoplasmic process encompasses material in the extracellular space. The matrix of the process contains many fine fibrils and therefore appears darker than the matrix of the adjacent cytoplasm. *N*, nucleus. × 33,000.




Figures 39 to 41. Examples of cytoplasmic bodies most often seen at the periphery of the Golgi areas in late stationary phase fibroblasts. Fig. 39, × 38,000; Fig. 40, × 44,000; Fig. 41, × 66,000.

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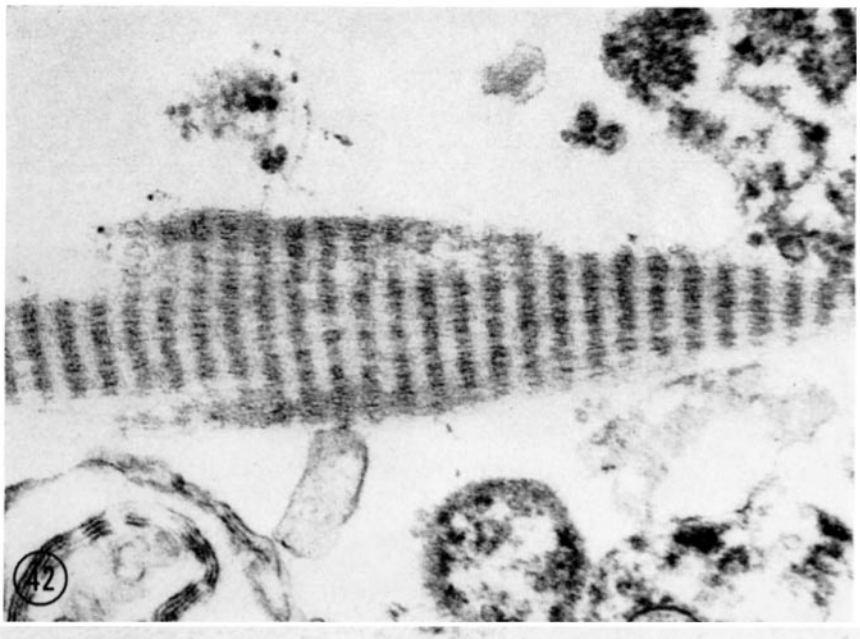
FIGURE 42 A type of extracellular fiber produced by established fibroblast lines. The period measures approximately 850 A. The width of the intraperiod dark band averages 400 A, that of the light band 450 A. × 51,000.

FIGURE 43. A higher resolution study of the fiber of Fig. 42 indicates its fibrillar substructure and demonstrates discontinuities in the intraperiod dark band. Fibrils 40 to 70 A thick are seen in the dark bands. Fibrils approximately 40 A thick (arrows) appear to occasionally traverse the amorphous light bands. × 140,000.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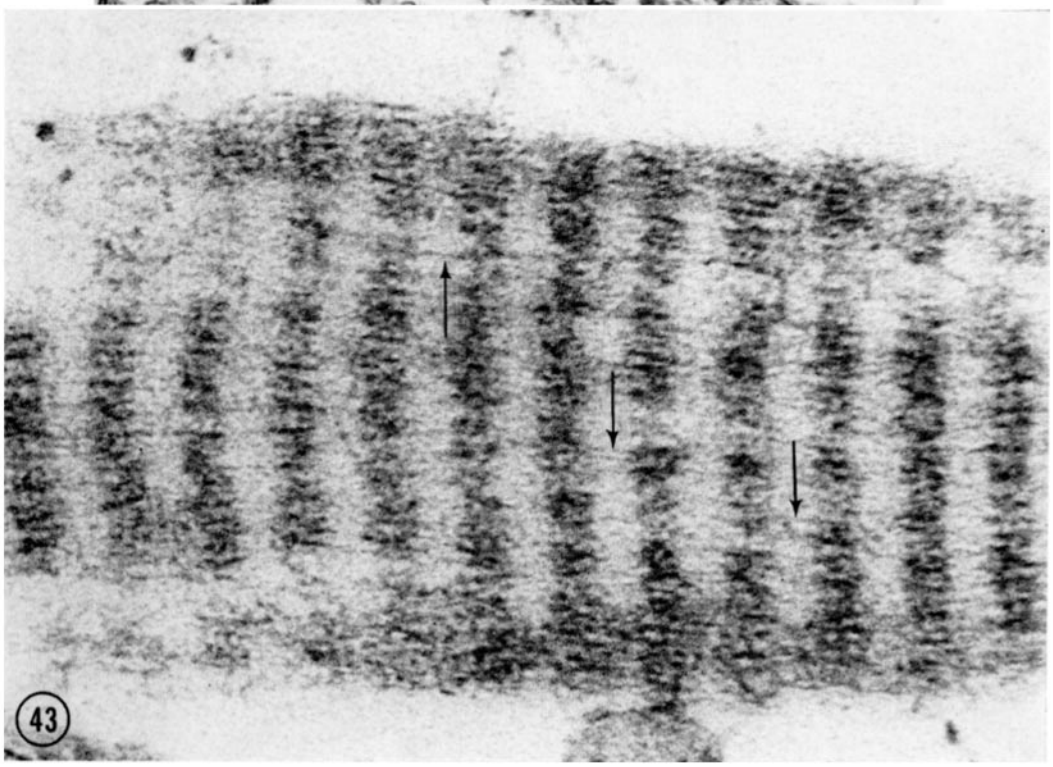
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