CELL FINE STRUCTURE AND BIOSYNTHESIS OF INTERCELLULAR MACROMOLECULES

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ABSTRACT *Fibroblasts* active in collagen production show a rich development of the endoplasmic reticulum (ER) and an enlarged Golgi complex, both characteristic of cells engaged in protein synthesis. The relatively quiescent *fibrocyte*, on the other hand, is deficient in these same cytoplasmic systems. When fibroblasts (or chondroblasts) are provided with tritiated (H^3) proline, the label shows, by autoradiography, incorporation first into materials (collagen) in the cisternae of the ER, transfer thence in time to the Golgi, and eventual secretion into the extracellular environment. $Sulfur^{35}$ incorporation into chondroitin sulfate appears to involve only structural elements of the Golgi complex. There is increasing evidence of intimate fibroblast (or cell) involvement in the initiation and orientation of unit collagen fibrils. This question is reexamined in relation to the development of the prominent basement lamella of young adult lampreys.

INTRODUCTION

It is now generally agreed that the macromolecular components of collagen are made in large part by a family of mesenchymal cells which include fibroblasts, chondroblasts, and osteoblasts. This belief is based on several direct observations of a close relation between, for example, fibroblasts and collagen during normal morphogenesis and wound healing and the production of collagen and mucopolysaccharides by fibroblasts *in vitro* where no other source of these materials is evident. The exact mechanism by which the cell makes this contribution to its environment has long been discussed and debated, but in spite of all this thought and effort it persists as one of the outstanding problems of collagen and connective tissue research. For purposes of discussion, the question may be variously subdivided and defined. One may ask whether the fibroblast possesses and uses the same mechanisms of synthesis and secretion found in other protein-synthesizing cells, that is, the ergastoplasm-Golgi association and sequence. Or does the fibroblast also donate materials to its environment by shedding sections of its cortex and adjoining cytoplasm through a kind of apocrine secretion? Or is it possible that this cell uses both devices? These are questions of first importance, especially

in understanding the role of the fibroblast in connective tissue formation and the various pathologies of connective tissues. As part of these, there are several phenomena that especially interest students of development. The patterns, sometimes extraordinarily precise, which collagen fibrils and bundles assume in particular tissues, seem to demand in their production an intervention of equally ordered patterns of cells in tissues, although there are those who claim that strictly acellular ordering of the macromolecules involved in a framework of organization in the ground substance could account for all one finds. The striking diameter differences in mature collagen fibrils found in different tissues argue for differences in the "core" structure of the fibrils, differences which could limit the amount of tropocollagen incorporated in the growth of the mature fibril.

In this report some, but not all, these questions will be reexamined in the light of a few recent accomplishments of investigators using the electron microscope and associated autoradiography. I propose first to explore briefly the fine structure of the fibroblast and fibrocyte. From this observation, we may decide whether and to what extent these cells have the submicroscopic machinery usually used in protein synthesis. Then I should like to introduce some new and valuable observations derived from autoradiographic studies of other investigators. And finally, we may take another look at the relationship of cell to collagen production and consider the factors which could influence the organization encountered in such famous ordered systems as the subepidermal basal lamella found in amphibia and fishes.

1. *The Fine Structure of the Fibroblast and Fibrocyte*

The fibroblast is, of course, a familiar cell in developing connective tissue, whether this is part of a repair process or part of normal embryogenesis. It is usually described as a fusiform cell, but when viewed from other directions it may appear stellate and decidely thin and extended. In its staining properties it is basophilic, as are other cells active in reproduction or in the synthesis of protein. The nucleus is oblong as a general rule, and the cytoplasm seems relatively abundant. Mitochondria are small and not impressively numerous. Usually the outlines or limits of a fibroblast involved in collagen genesis are indistinct. This is the general light microscope picture of this active and possibly proliferating cell.

The fully differentiated, relatively inactive, fibroblast—to be called here *fibrocyte*—is different in several respects. It may be extremely tenuous. The cytoplasm, which in volume is greatly reduced, stains less prominently but has more distinct limits. In other respects the light microscope picture is not very informative.

These differences and others find an exaggerated expression in the image provided by electron microscopy. The fibroblast appears as a cell with an abundant cytoplasm, embedded in varying amounts of fibrous material (Fig. 1). Its cytoplasm shows numerous profiles of rough surfaced cisternae which make up a fully developed ergastoplasm or endoplasmic reticulum typical of protein-producing cells

(1). The ribosomes attached to the membrane surfaces are prominent and arranged in only a few patterns or polyribosome aggregates. Other clusters of ribosomes are present in the cytoplasmic ground substance, engaged presumably in the synthesis of enzymes and structural proteins for use in this part of the protoplast. The Golgi component is characteristically prominent. Thus the average thin section of a fibroblast usually includes several groups of flattened, closely arranged cisternae with a large number of satellite vesicles clustered about. This picture, though of course, completely static, is descriptive of great activity. Details of associations between elements of the ergastoplasm and Golgi, and likewise between types of satellite vesicles and the Golgi, can be made out (see descriptions by Revel and Hay (2)), but with so little known of their significance it is perhaps not appropriate in this particular instance to describe them.

The nucleus of the fibroblast may appear smooth or somewhat lobate (Fig. 1). The condensation of chromatin around the margin is not prominent. Instead, the particulate elements, *i.e.* chromatin filaments, are more diffusely organized than are the equivalent components of the chromatin picture in the mature fibrocyte (Fig. 2). Here again the appearance is reminiscent of a growing and metabolically very active cell as opposed to that of a quiescent, relatively inactive one.

The cell surfaces will be discussed later when consideration is given to the fiber-cell relationships. At this juncture, it is sufficient to comment that the surface of the fibroblast appears irregular and indistinct. In part, one can relate this appearance to the fact that along any one surface the plasma membrane frequently changes orientation with respect to the plane of section. This does not serve in all instances as an explanation, however, and there are places where the membrane of the cell fades into the adjacent intra- and extracellular materials or is definitely interrupted in its continuity.

The fibrocyte, which inhabits relatively mature and stable connective tissue, is, compared to the fibroblast, a simple cell. The volume of the cytoplasm is strikingly reduced, hardly covering the nucleus in places (Figs. 2 and 3). Profiles of the ER cisternae with particles attached are few; the Golgi component is insignificant to the point of vanishing. A few free ribosomes remain. The ground substance has a finely fibrous texture. The nucleus is characterized by a pronounced condensation of chromatin just beneath the envelope, and pores in the envelope are present but infrequent.

The surface of this cell seems relatively regular and intact (Figs. 2 and 3). The plasma membrane is easily followed as a distinct limiting line or boundary. There are more cortical vesicles or pits than in the fibroblast, but otherwise the surface seems relatively inactive.

Small, slender, or extremely thin extensions of fibroblasts infiltrate the collagen of the surrounding extracellular environment to a greater extent than had been anticipated. Some of these are not more than 30 to 40 mμ in width, extremely

slender, and too small to be resolved by light microscopy. As shown in Fig. 3, these may reach out to contact another cell of the same type and so form a continuous cellular tissue. The picture suggests that in normal mature connective tissue the fibrocyte population in any one region forms sheets or cords of contiguous cells with large intercellular spaces filled partially with collagen fibrils.

2. *The Fibroblast and Collagen Production*

There is, of course, ample evidence that the fibroblast and closely related cells of mesenchymal origin are the source of collagen and associated materials of the connective tissue. The constant presence of these cells at the site of and during the phases of collagen (and cartilage) production makes this fact self-evident. It matters not whether the system under observation represents *in vivo* growth or repair (3-6) or *in vitro* culture of connective tissue (7, 8), the newly formed collagen always appears in association with fibroblasts or related cells. This obvious dependence notwithstanding, it is of some value to look at supplementary information supporting the fibroblast-collagen relationship.

The fine structure of the fibroblast, reviewed just above, describes it as a cell active in synthesis and secretion, for cells engaged in protein synthesis are consistent in showing a pronounced elaboration of the rough form of the endoplasmic reticulum (ER) or, by another name, ergastroplasm (9). The product of synthesis accumulates in the cisternae of the ER and thence finds its way to a hypertrophied Golgi where it is packaged for secretion. In this latter regard as well, the fibroblast (and chondroblast) fits the repeatedly demonstrated picture. The fibro*cyte*, on the other hand, which leads a relatively dormant life in mature connective tissue, is notably deficient in both these systems of ultrastructure. Hence it is at least corroborative of the accepted fibroblast role in collagen production to find the cell possessing the morphology of a secretory unit.

Of no less significance in this regard are accumulated observations on the secretory products of fibroblasts and related cell types kept for periods under conditions of tissue culture. For example, fibroblasts from explants of chick embryo heart tissue have been shown by Kuwabara (10) to yield per cell a twofold net increase in hydroxyproline-containing protein over a period of 14 days. Similar studies by Jackson and Smith (11) on osteoblasts under *in vitro* culture describe a similar production of collagen protein identified on the basis of hydroxyproline content. These investigators make the interesting point that monomeric collagen appears in such cultures in advance of microscopically identifiable collagen fibrils. It would appear, therefore, that the fibroblasts are actively secreting molecular collagen before they have differentiated far enough to initiate fibril production. This point will be touched on again below. We find it important to note here also some results of Grossfield *et al.* (12) from similar experiments with fibroblasts of human embryo skin and bone. Unlike the former studies, these focused on the production

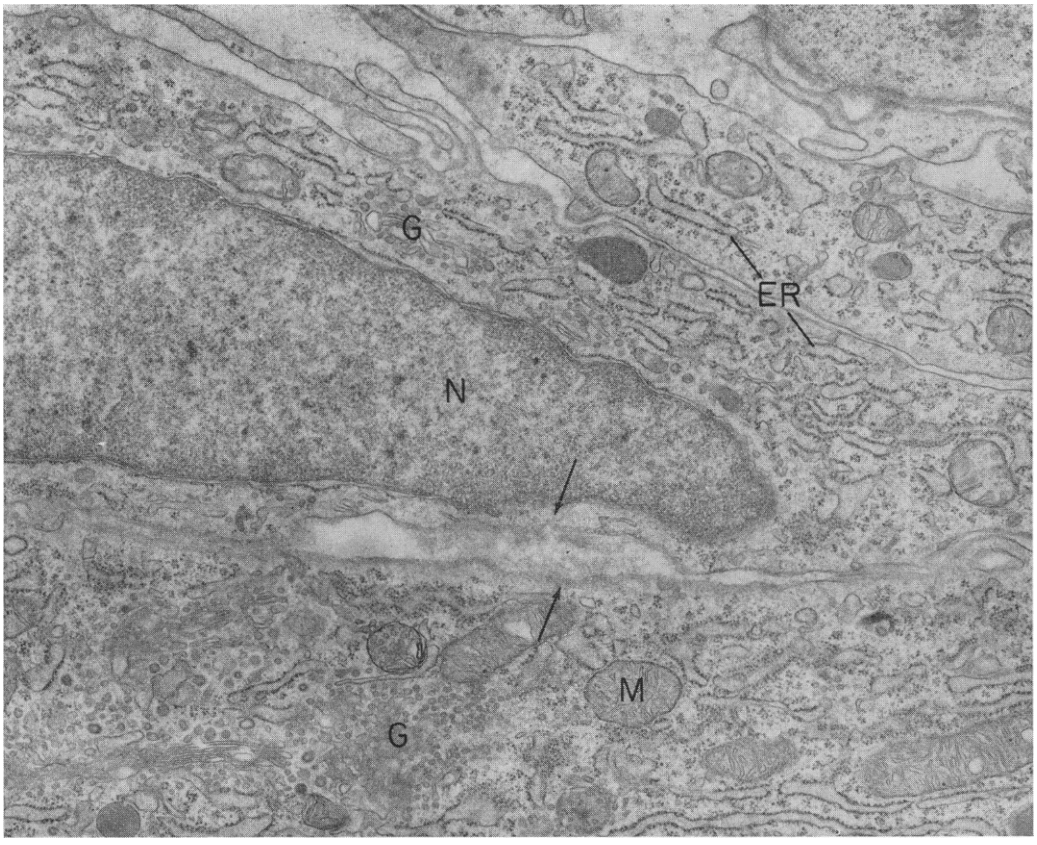


FIGURE 1 Micrograph showing a group of *fibroblasts* associated with stroma development in a transplantable hepatoma. Such cells, active in collagen production, show an abundant cytoplasm which contains a richly developed ergastoplasm (*ER*), numerous free ribosomes and polyribosomes, and a greatly hypertrophied Golgi (*G*). The chromatin in the nucleus (*N*) is concentrated in relatively diffuse masses beneath the envelope. Condensations of finely fibrous material are distributed over and closely adherent to the cell surface (at arrows). The texture of this is duplicated by material within the cell cortex. Images of the cell at higher magnifications may show extremely fine beaded fibrils (50 to 100 A in diameter) closely applied to its surface. Mitochondria (*M*) are neither numerous nor unusual. × 18,500.

of mucopolysaccharides and demonstrated that such cells actively secrete hyaluronic acid and, to a lesser extent, chondroitin sulfate. In any attempt, therefore, to correlate the morphological aspects of the fibroblast with its functional properties, one should think in terms of more than collagen production alone. This is especially true for the chondroblast, which plays an exaggerated role in the production of extracellular ground substance and a relatively minor one in collagen synthesis (6).

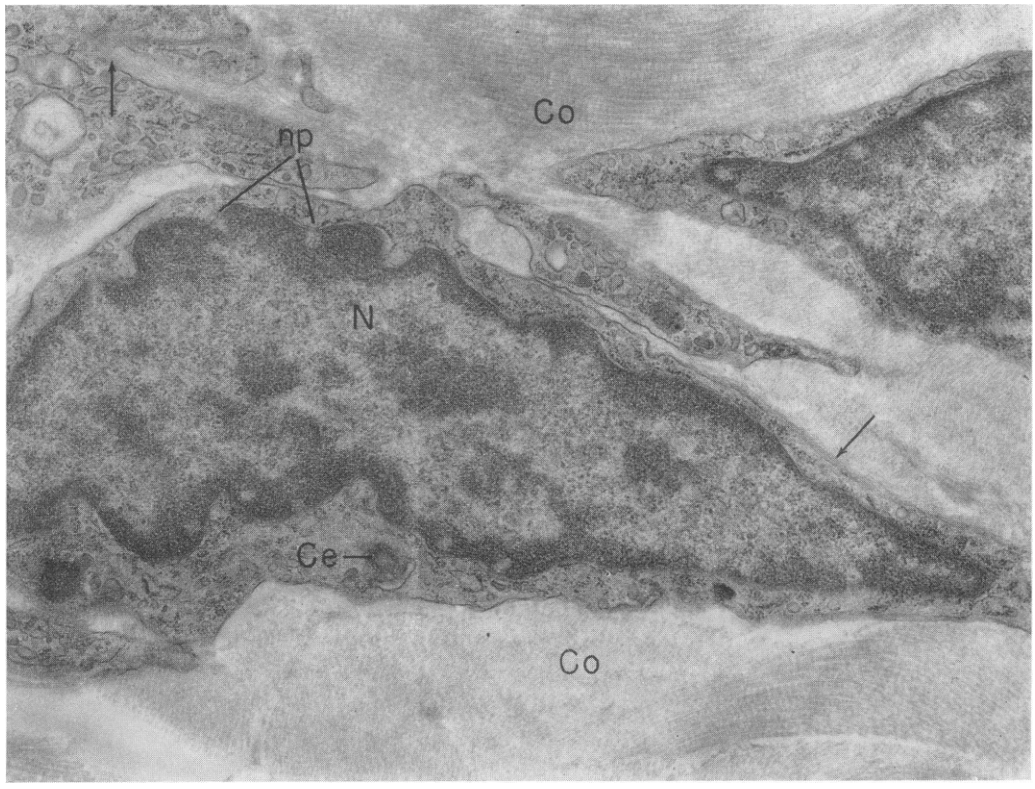


FIGURE 2 This micrograph depicts a mature *fibrocyte* as found in the dermis of the bat. As typical for these relatively inactive cells, the volume of cytoplasm is less than occurs in fibroblasts. There is little evidence of ergastoplasmic or Golgi components in the cytoplasm. A centriole, one of two, is apparent at *Ce*. The nucleus (*N*) is marked by pronounced condensations of marginally located chromatin. Only a few pores (*np*) are present in the envelope. The plasma membrane is continuous, and its outside surface is coated with a thin fibrous mat in which the component filaments are extremely fine (arrows) (*ca.* 100 A). They coincide in appearance with more randomly arranged fibrous elements in the cell cortex. They are much finer than the mature collagen fibrils (*Co*) which fill the intercellular spaces. × 15,000.

3. *The Intracellular Site of Collagen Synthesis*

It is fortunate for our current understanding of the biosynthesis of such macromolecular materials as collagen that the techniques available for the structural-functional analysis of cells have increased greatly in resolution. Thus we are increasingly able to pinpoint within a cell the places of synthesis and segregation of collagen (and other proteins) for secretion, and the organelles and subcellular systems involved. However, even before these techniques were developed, there were reports of granules in fibroblasts having specific staining properties which

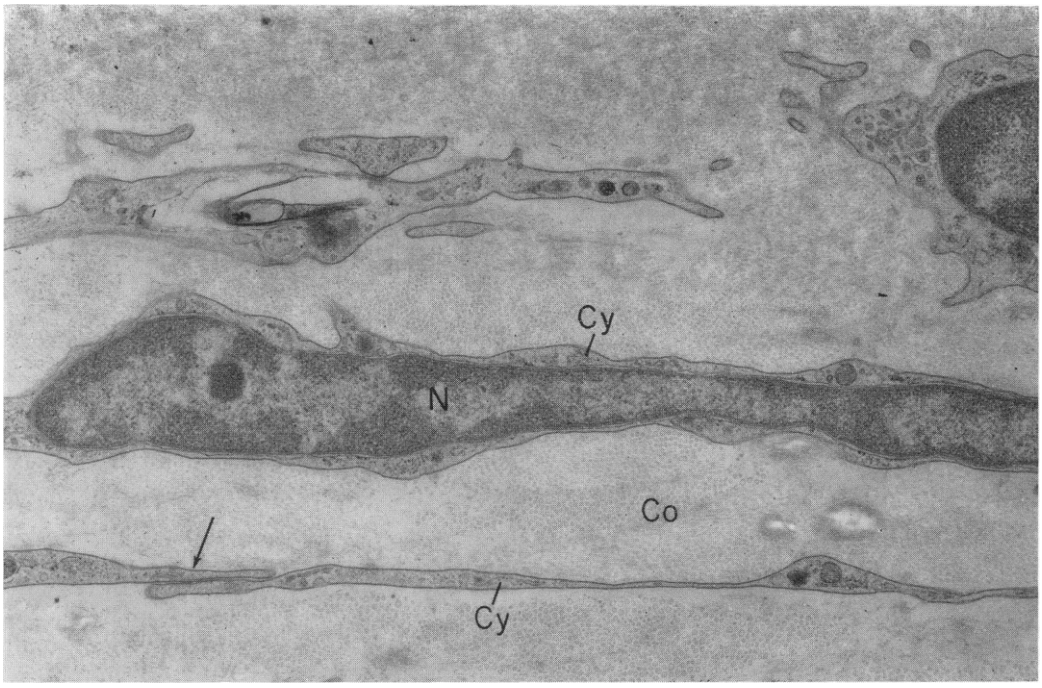
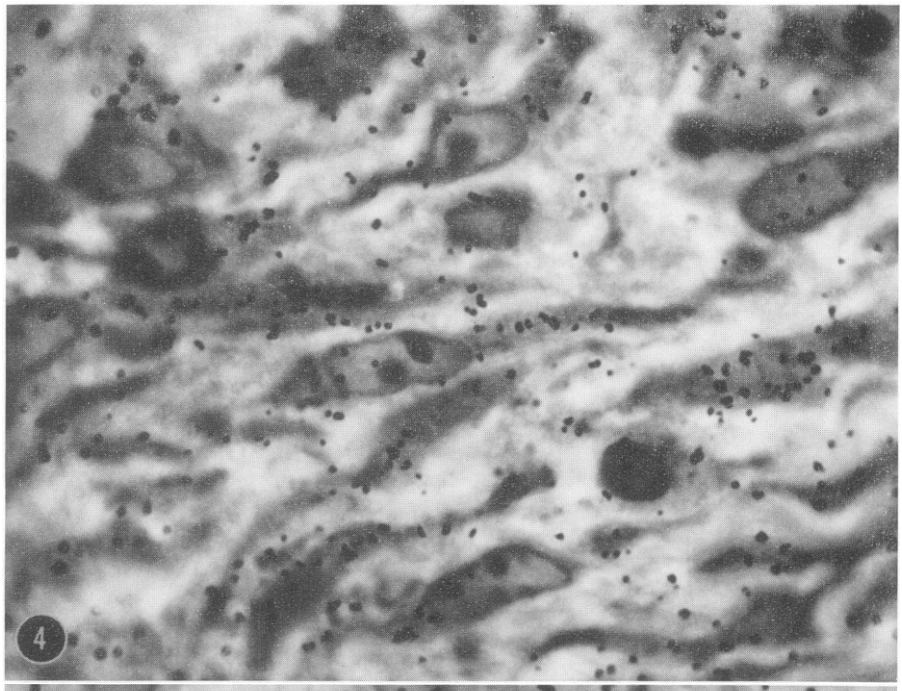


FIGURE 3 Here the picture includes fragments of "fibrocytes" associated with the perichondrium of tracheal cartilages in the bat. These are very thinly extended cells. The nucleus (*N*) is limited by the usual envelope forming a boundary with a very thin layer of cytoplasm (*Cy*). Such cells as these possess slender marginal extensions which penetrate the collagen masses (*Co*) and reach out to touch other cells of the tissue (arrow). × 17,500.

related them to collagen and its intracellular production. At first observed in living cells (3, 4), they were later identified with collagen on the basis of similar staining properties (13). Subsequent to this, the PAS-positive character of these inclusions was emphasized in relating them to mucopolysaccharides and the production of extracellular ground substance (14). And in a study of osteoblasts, Sheldon (15) noted a coincidence between the number and size of these PAS-positive inclusions and the dilated cisternae of the ER and decided that the two should be equated, an interpretation that was given further credence by Cameron (16) in a related study of osteoblast fine structure. Thus certain crude correlations were established between fibroblast fine structure and the production of connective tissue components.

These have since been extended and sharpened. For example, Lowther *et al.* (17) have followed the incorporation of C^{14}-labeled amino acids into the cells of carrageenin-induced granulomata by examining cell fractions of these isolated by centrifugation. They found that collagen associated with the microsomal fraction,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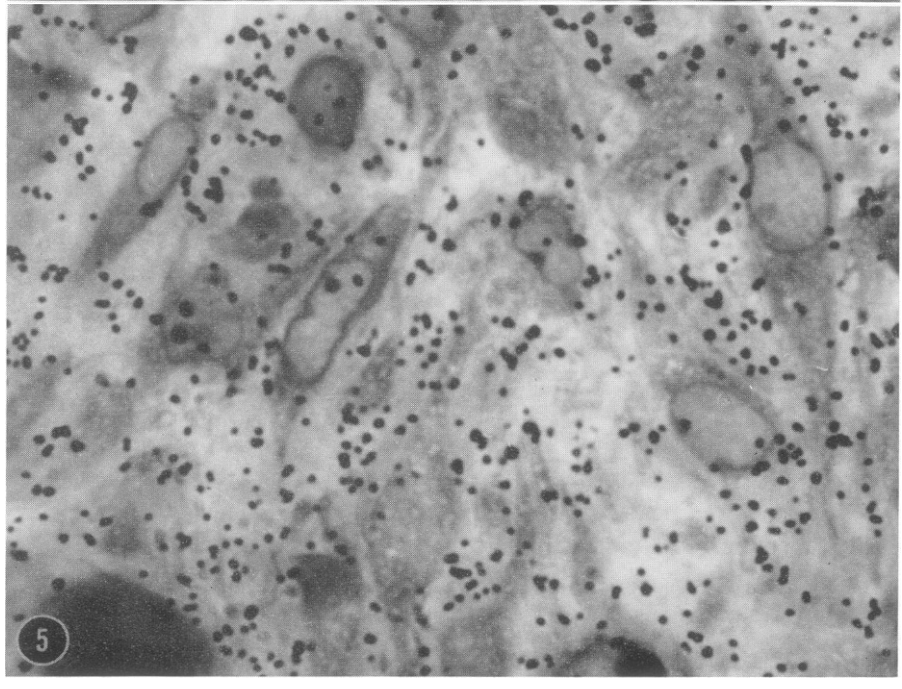
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consisting of demonstrable fragments of the ER, possessed the highest radioactivity. Though this information is in no way surprising in the light of all other evidence pointing to ER involvement in protein synthesis (18), it does nonetheless establish more definitely the association of the rough-surfaced endoplasmic reticulum with the synthesis as well as the segregation of collagen. The authors (17) make the additional point that the collagen or tropocollagen present in the microsomal fraction is easily extracted with neutral salt solutions and thus resembles the monomeric form abundantly present in the extracellular spaces of embryonic and newly forming connective tissue (19). These and other investigations describe the uptake and incorporation of amino acids into collagen *via* the microsomes. Presumably the synthesis is achieved by polyribosomes and associated messenger-RNA as in other systems of this kind currently investigated (20).

The progress of these metabolites through the cell's multifarious structures can be followed as well by autoradiography and electron microscopy. The values of these combined techniques for increasing the resolution and identifying more precisely the location of the labeled materials have been demonstrated by Caro (21) in a study of the uptake and incorporation of tritiated leucine into pancreatic zymogen. Using essentially similar procedures, Ross and Benditt (22) have recently followed the uptake of proline-H^3 into fibroblasts of healing wounds in normal and scorbutic guinea pigs. This amino acid, known to be used in collagen synthesis, was rapidly assimilated by cells in the repairing tissue and achieved a maximum concentration within an hour after injection. For the most part the silver grains marking its location appeared over the cytoplasm. Later, but within 24 hours of injection, it departed the cells and lodged in the surrounding collagen. These results at the light microscope level are illustrated in Figs. 4 and 5 (which, along with the micrographs in Figs. 6 to 8, were generously provided by Dr. Ross and Dr. Benditt). Electron microscopy of parallel preparations further resolved

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FIGURE 4 Photomicrograph of fibroblasts in region of healing wound in dorsal skin of guinea pig. Thirty minutes before this tissue sample was taken and fixed, the animal was injected with a heavy dose of tritiated proline (proline-H^3). Sections coated with photographic emulsion (autoradiographs) showed upon development an accumulation of silver grains over the cells and far fewer over the intervening collagen and ground substance. This evidence points to a rapid uptake and incorporation of the labeled material. The resolutions available in this preparation are capable of little more than revealing a preponderance of grains over the cytoplasm. × 1,600.

FIGURE 5 The tissues and techniques are similar here to those used for the preparation depicted in Fig. 4. The single difference is that the tissue was taken 24 hours after the proline-H^3 was injected. As a consequence of the longer time lapse, the label (indicated by silver grains) is very largely over the intercellular, collagen-filled spaces where it has moved from the fibroblasts. × 1,600.

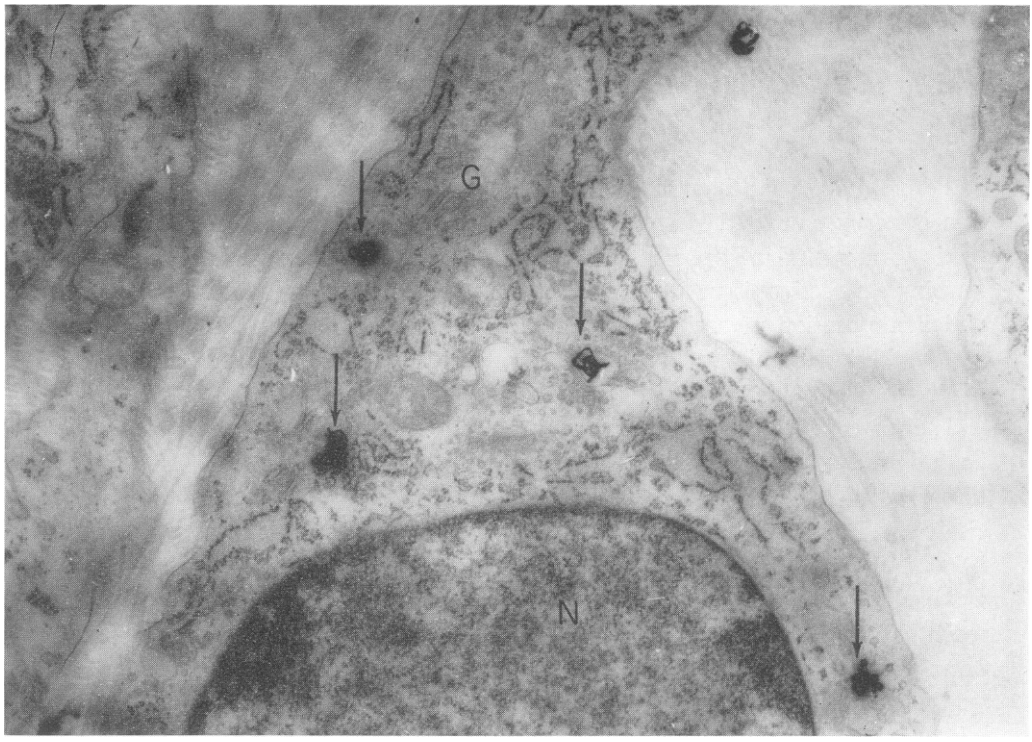


FIGURE 6 This depicts in an electron micrograph a fibroblast from the same healing wound which was the source of the low power light preparations shown in Figs. 4 and 5. The important difference here is that the resolutions provided by the EM help to relate the silver grains (arrows) to a particular intracellular structure. In this instance, each grain is over elements making up the Golgi component (*G*). Only one grain in this image is outside the cell and may be a product of background radiation. × 18,500.

the distribution of the label. As illustrated in Fig. 6, representing a tissue sample taken 1 hour after injection of label, the silver grains (marked by arrows) are localized over the Golgi complexes in the cytoplasm. Thus, if protein synthesis in fibroblasts follows the pattern found in other cells, the protein-bound proline is within 1 hour being packaged for secretion. The involvement of the rough ER in the process is indicated by the location of the silver in Fig. 7. This is from another cell in the tissue sample also taken 1 hour after injection. Obviously the grains result from emissions originating in material within the dilated cisternae, so that at some point in its synthesis the proline- or hydroxyproline-containing collagen is within these elements of the ER. The eventual location of the labeled product is shown in Fig. 8, which represents the distribution 24 hours after injection. Clearly by this time the majority of the grains are distributed over the intercellular spaces rich in collagen fibrils.

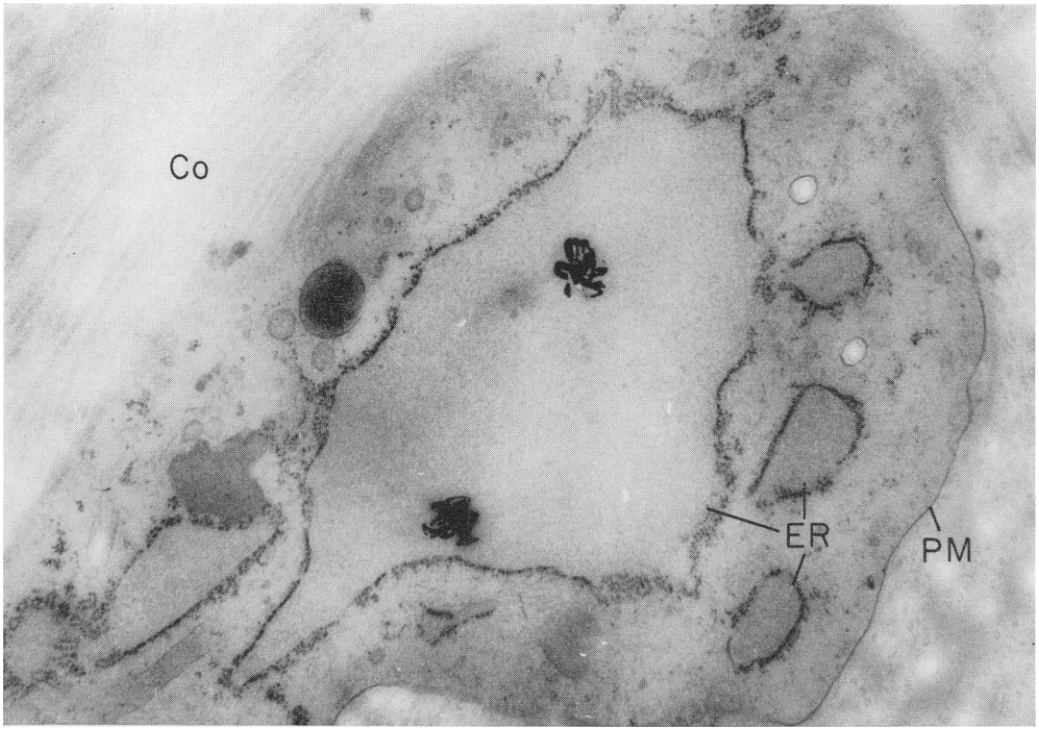


FIGURE 7 Here the image includes a small part of a fibroblast containing a dilated cisterna of the *ER;* this is from a healing wound of the guinea pig. The dense silver grains describe the presence of the weak beta emitter (H^3) in the material within the cisterna and not especially elsewhere. This is from a sample taken 1 hour after injection of the animal with proline-H^3. The plasma membrane (*PM*) is distinct on one side where it is oriented normal to the section. × 21,500.

In so far as their results concern the synthesis and secretion of collagen, Revel and Hay (2) in a report on chondrogenesis describe a course of events similar to those depicted by the fibroblasts in the Ross-Benditt experiments. Tritium-labeled proline was injected in *Ambystoma* larvae in which limb regeneration was in progress. Within 10 to 13 minutes after injection labeled material appeared in the ergastoplasm (cisternae of rough ER), within 20 to 30 minutes in the vesicles of the Golgi complex, and after 1 to 2 hours in the cartilage matrix. Their illustrations include conclusive evidence of the labeled product leaving the cell by a fusion between the Golgi-derived secretory vesicles and the cell surface (Fig. 9). The subsequent migration of the labeled product in the cartilage matrix is striking, if, as they claim, the product is the highly asymmetric molecule of tropocollagen. From this assumption and their observations they reason soundly that tropocollagen may move several microns from the cell before it is incorporated into fibrils. Other sites of proline-rich materials were not noted in the chondrocytes

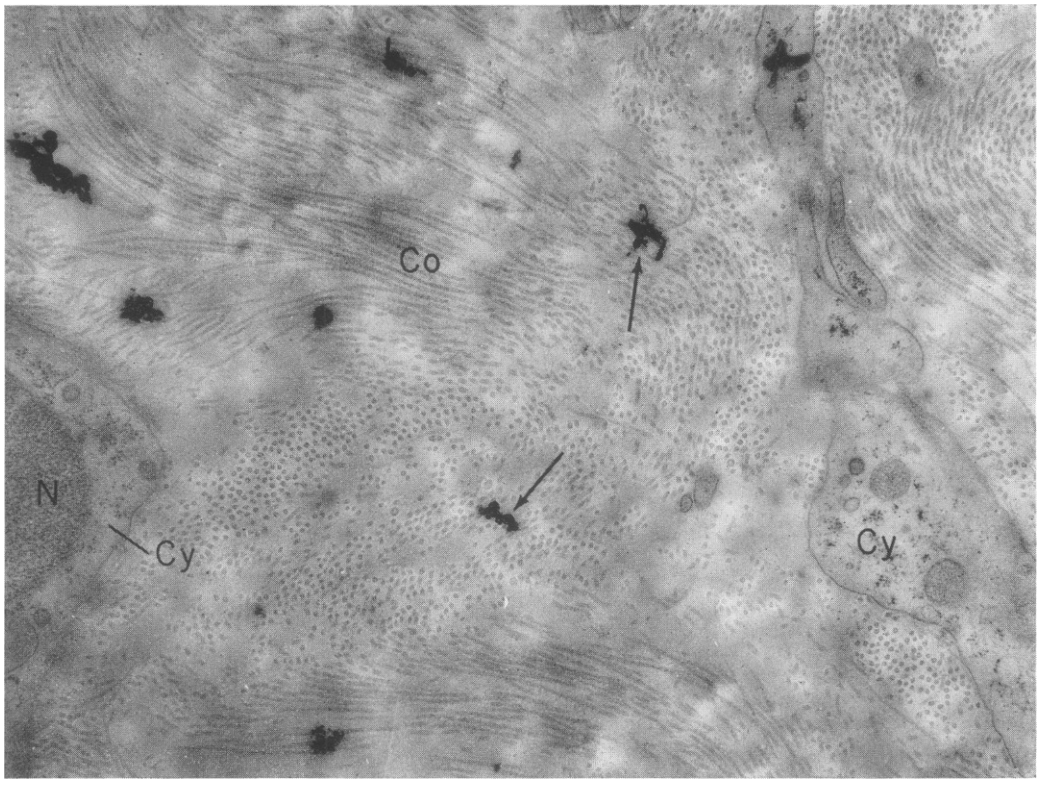


FIGURE 8 Electron micrograph of tissue from the same wound as that from which preparation was made for Fig. 5.

Electron micrograph of wound tissue showing large area of collagen fibers and marginal portions of two cells (*Cy*). This sample, like that depicted in Fig. 5, was taken from guinea pig 24 hours after injection of proline-H^3. By this time most of the labeled collagen, as shown by silver grains (arrows), has moved into the intercellular spaces. × 19,000.

Figs. 4 to 8 inclusive were generously provided by Dr. R. Ross and Dr. E. P. Benditt.

studied. Whether the sample was large and contained chondrocytes in various stages of differentiation was not indicated.

Here then is another elegant demonstration of a pathway of synthesis and secretion used by a cell of the connective tissue family for the production of a proline-rich protein. When considered in conjunction with some recent findings of Sheldon and Kimball (23) of striated fibers (long spacing about 2,000 A) within Golgi vesicles, one is left with no reason to doubt that this ER-Golgi combine is the major machinery involved in the biosynthesis of collagen.

We should not leave this discussion without at least brief mention of the uptake and binding of radiosulfate into chondroitin sulfate by chondrocytes. A more

extended review may be found in the paper by D. D. Dziewiatkowski within this volume. In a paper in press, Godman and Lane (24) have reported on the accumulation of the S^{35}-rich compound in microvesicles of the Golgi complex within 3 minutes after presentation to the cells (Fig. 10). From these it enters larger vesicles, possibly resulting from fusion of the smaller, and is from these secreted. One would reasonably conclude, therefore, that while this important component of the cartilage matrix is, like collagen, assembled in Golgi-associated vesicles, it takes a shorter route in getting there and bypasses the ergastoplasm completely.

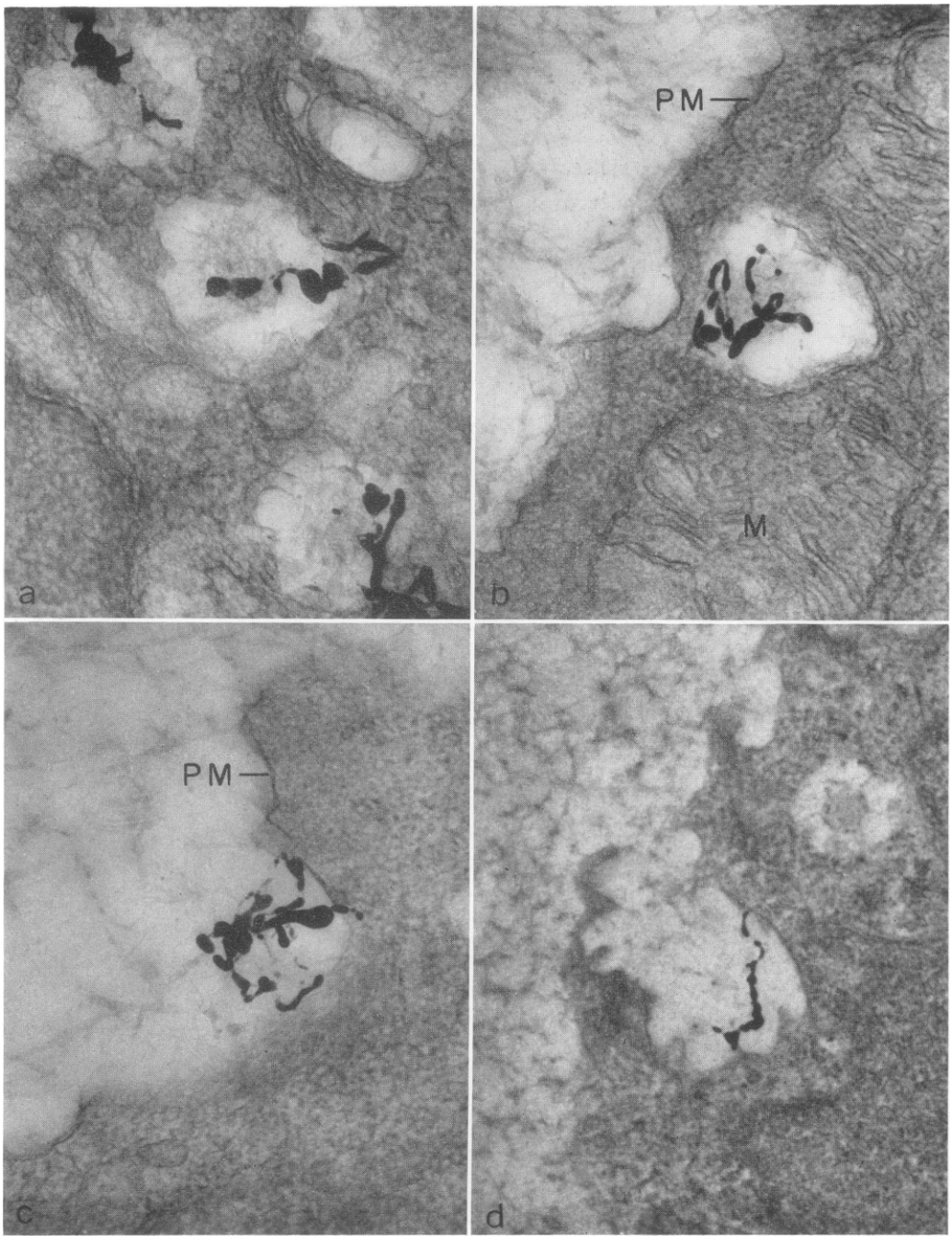
These studies using autoradiography and electron microscopy give their impressive evidence of the route by which some components of cartilage, namely collagen and mucopolysaccharides, are contributed to the extracellular matrix. Morphological studies of the development of cartilage illustrate that in their differentiation to chondrocytes chondroblasts go through various phases, in some of which they appear to lose a variety of cortical components including fine filaments. The fate of these is not known, but it has been suggested that here, as apparently in fibrocytes, they constitute the primary fibrils upon which tropocollagen condenses or polymerizes to form the larger fibrils of mature collagen (6). That the above reports on primary investigations with labeled precursors have not shown any association of label with any structure except Golgi vesicles (or ER cisternae) preliminary to release from the cell can be interpreted to mean either that other cortical materials released contain no collagen or sulfonated compounds or that the cells studied were not at the time of fixation active in anything except merocrine-type secretion of Golgi-assembled products.

4. *The Deposition of Collagen*

We have just considered secretion of the merocrine type by which tropocollagen is made available to the intercellular spaces and environment. The process in morphological terms is the same in both fibroblasts and chondroblasts and probably in all cells of this type. A Golgi-derived vesicle migrates to the cell surface, fuses with it, forms an opening or stoma, and discharges its contents. These diffuse away to be incorporated into the growing fibrils of collagen in the surroundings. This story seems well established, especially from the recent work of Revel and Hay (2) and of Ross and Benditt (22).

But is this the only manner in which a fibroblast contributes of itself to its environment? This question will remind some readers of a debate which has extended over several decades. Where do collagen fibrils have their origin—in the cell, at its surface, or at some point removed from the cell? It is probably not important here to review this argument in all its details, but I would like to recall a few observations which suggest at least that the fibroblast contributes to collagen formation by more than one type of secretion.

The *in vitro* culture of fibroblasts is a convenient and rewarding method for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observing cell-fiber relationships during collagen production. Demonstrated as a useful device for the purpose by M. R. Lewis in 1917 (7), it has since been used repeatedly with the same end in view (8, 25, 26). There is no doubt that under the conditions of culture, fibroblasts will and do produce mats of collagen over the coverglass or surface supporting the culture. Such mats are essentially laid down to wall off a foreign body represented by the coverglass. The interesting point to note from this system is that the fibrils of collagen form only over and among the cells and not out in the drop of culture medium even though that is, with respect to the cells, in a hanging drop position. Furthermore, fibers never appear on the culture surface beyond the margin of advancing cells. Instead, they appear from electron micrographs to have their origin at the cell surfaces and most especially over marginal or cortical thickenings in the cell, which if observed by light microscopy would be referred to as stress or fibroglial fibers (Figs. 11 and 12). After such primary fibrils are separated from the cell surface, they increase greatly in diameter by accretion of soluble collagen on their surfaces. The primary fibril seems, however, to be a product of the cell surface or the cortex beneath the surface (8, 25, 26) The shedding or ecdysis or excortication of fibrous elements is difficult to observe in cultured units or in any fixed material for that matter. The eventual picture is static and lends itself to several interpretations and prejudiced concepts. However, several observers, of whom some are objective, have now noted discontinuities of the plasma membrane over fibrous regions in the cortices of fibroblasts active in collagen deposition under *in vivo* as well as *in vitro* conditions (26-29). The same phenomena have been noted at the surfaces of chondrocytes during stages of active cartilage formation (6). Illustrations of the appearance of fibroblast surfaces thus interpreted are shown in Figs. 13 to 15.

There seems little reason to regard the conditions of tissue culture as being so unusual as to engender some abnormal sequences in collagen production. The environment is probably not worse physiologically than that encountered in many situations of tissue repair. Nor does it seem appropriate in the face of the evidence of cell involvement to invoke some mysterious and assumed capacity of the fibro-

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FIGURE 9 This series of micrographs is presented to show Golgi-derived vesicles in various phases of collagen secretion from chondrocytes. The images include margins or near margins of chondrocytes from regenerating cartilage of an *Ambystoma* larva. The animal had received a pulse of proline-H^3 30 or more minutes before the tissue was fixed. The label, marked by silver grains, indicates the presence of collagen. The micrograph at (*a*) includes Golgi vesicles containing labeled protein; at (*b*) a similar vesicle is close to the cell surface; at (*c*) and at (*d*) this type of vesicle has fused with the plasma membrane (*PM*) from the underside and is probably discharging its contents. A mitochondrion is indicated at (*M*). *a*, *b*, and *c*, × 47,500; *d*, × 23,500.

Micrographs courtesy of Jean-Paul Revel and E. D. Hay.

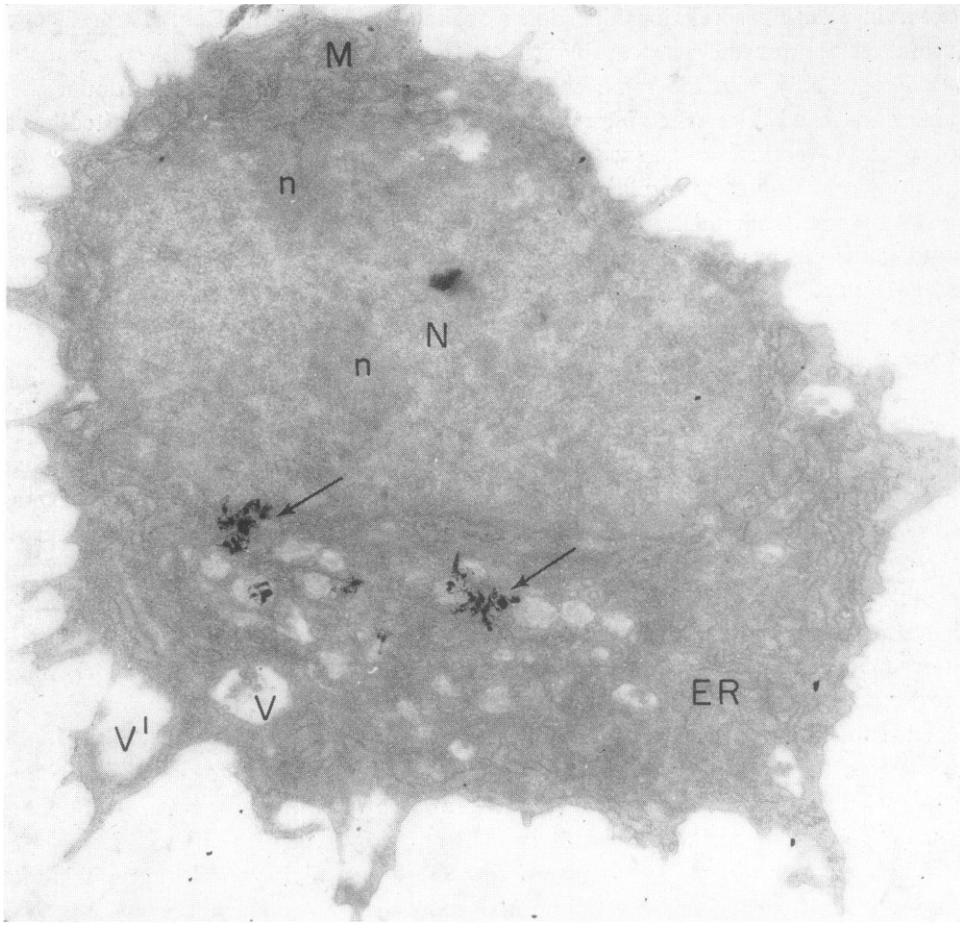


FIGURE 10 This image shows a chondrocyte from the femoral cartilage of a rat embryo fixed 10 minutes after a 5 minute pulse of sulfur[35]. The label, indicated by silver grains (arrows), is located in Golgi-associated vesicles and represents S^{35} incorporated presumably into chondroitin sulfate. These smaller units are believed to coalesce and form the larger units *V* and *V'* which are secreted. The endoplasmic reticulum is indicated at *ER*, mitochondria at *M*, nucleus at *N*, and nucleoli at *n*. × 24,000.

Micrograph courtesy of G. C. Godman and N. Lane.

blast to regulate the "chemical environment of the extracellular matrix" at distances of several microns from its surface in order to control the polymerization of collagen fibrils (2). If objections to culture-derived information are in order, one can turn to an examination of the same events in transparent chambers supported on the animal's surface. In a pair of classical papers which should be carefully read by anyone interested in this problem, Stearns (3, 4) describes collagen-fibroblast rela-

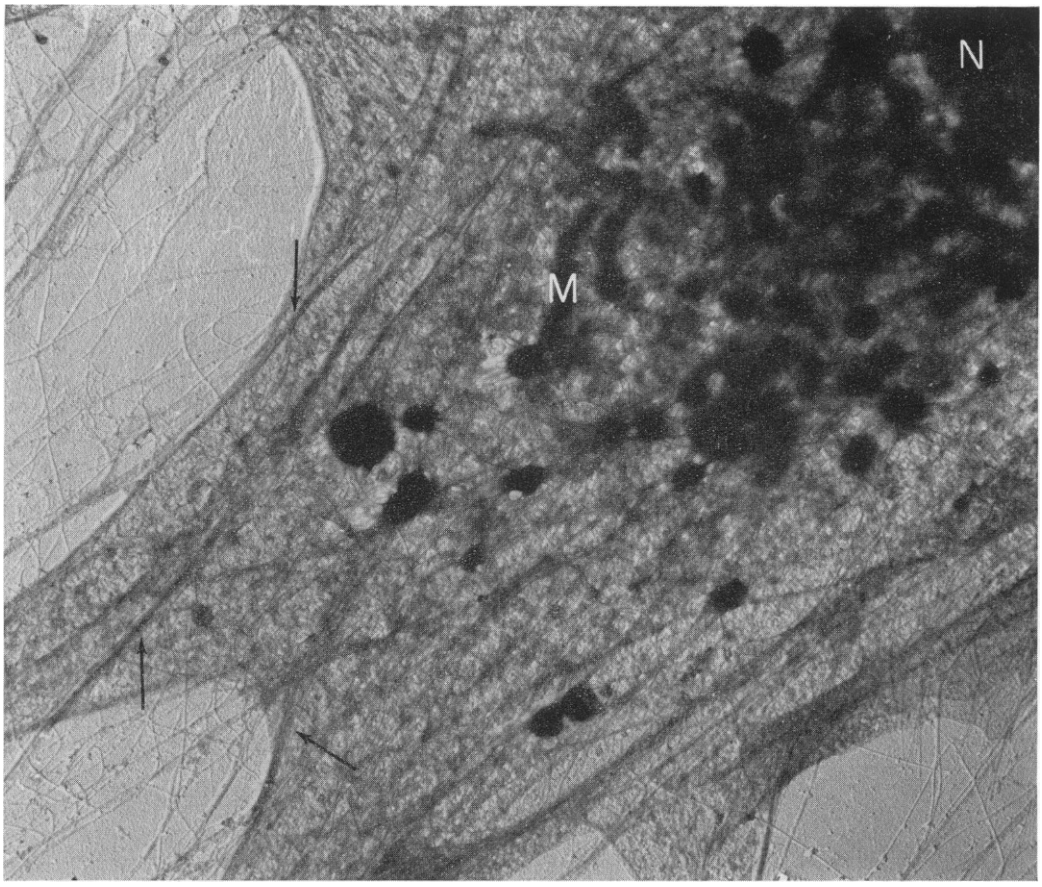


FIGURE 11 This includes part of a fibroblast cultured from an explant of skin taken from a 12-day-old chick embryo. This is not a thin section, but a part of a whole cell. The end of the nucleus (*N*) is at the upper right; mitochondria (*M*) are scattered through the cytoplasm. Small unit fibrils of collagen form a feltwork over the cell surface. Bundles of these (arrows) coincide with or extend from cell margins. A bundle of this type is shown to better advantage in Fig. 12. × 6,500.

tionships during tissue repair following the introduction of a chamber into the rabbit's ear. She makes the point that fibrils of collagen never appear in the wound in advance of the invading fibroblasts; "that this cell is intimately associated with the actual formation of the fibers. Furthermore, the orientation of these cells appears to influence the arrangement of the fibers." (3)

It seems inappropriate in this instance to review the question further. That fibroblast contact is essential to fibrillogenesis in connective tissue is indicated by the bulk of available evidence (26, 29, 37). And it seems illogical that this cell should depend on some ill-defined remote control for asserting its influence. Ob-

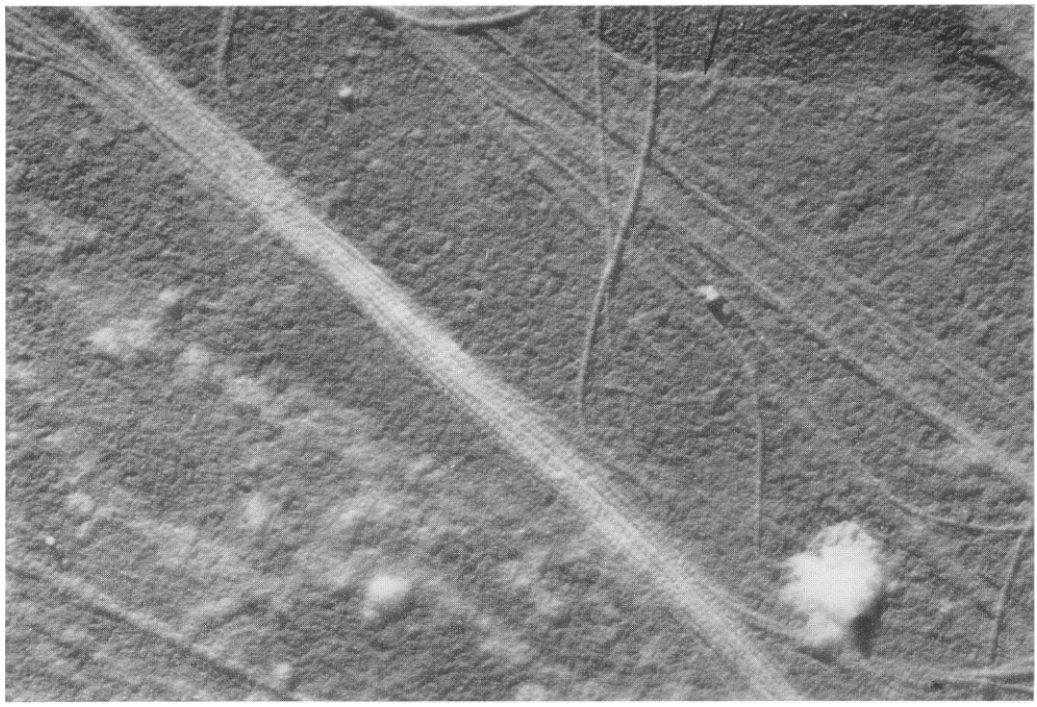


FIGURE 12 This shows a small part of the surface of cultured fibroblast including a bundle of unit fibrils of collagen. Some of these are continuous with the cell surface within the field of the image; others appear similarly attached to other points. A small stretch of the cell margin is included in the upper right (arrow). × 17,500.

viously the problem needs further study; there would otherwise be no excuse for debate.

One might assume that these capacities, first to put down an oriented primary or protofibril (in normal tissue development, or in repair, or in response to a foreign body), and second to secrete a material (collagen) which polymerizes out on the primary and contributes to its strength and survival would satisfy the recognized requirements demanded of fibroblasts. There are, however, observations which suggest additional secretory activities.

Stearns in the studies already noted comments extensively on the appearance at the cell's surface of vesicular masses which separate from the body of the cell. "This cytoplasmic material is apparently utilized in the production of fibrils, for it diminishes and eventually disappears as the fibrils form." (4) Similar excrescences can be seen in electron micrographs of fibroblasts engaged in collagen production (Fig. 16). They project from the surfaces of cell processes as in Stearns's images. The interesting thing to note from the electron micrographs is the presence of a row of vesicles across the base of the structure. This duplicates the pattern of

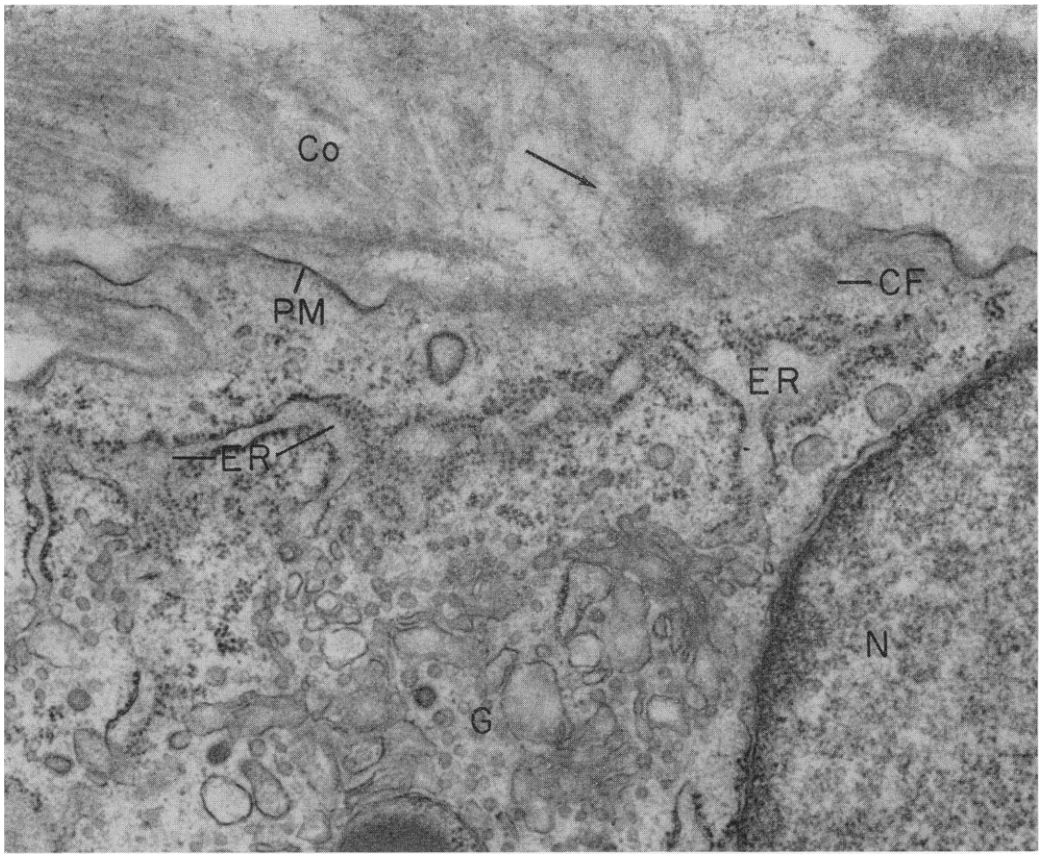


FIGURE 13 A part of the margin of a fibroblast from the stroma of a hepatoma. At points along the margin the plasma membrane (*PM*) is well defined; at other points the cortex and its dense fibrous content (*CF*) seem continuous with extracellular fibrous material of the same density. Collagen fibrils are indicated at (*Co*), the nucleus at (*N*), and the Golgi complex at *G*. × 34,500.

vesicles which appears in apocrine secretion recently noted by Parks (30). The vesicles in the layer or row are depicted as fusing to achieve the separation of the small cytoplasmic mass from the parent cell. A similar alignment of microvesicles around elements of the cytoplasm destined for sacrifices has been noted in chondroblasts during the development of femoral cartilage in embryo rats (6) and interpreted as a form of apocrine secretion. Following their separation from the parent cell, these excrescences, according to Stearns, gradually disintegrate; but in so doing they become foci of fibril development (4).

5. *The Post-Secretory Development of Collagen*

The progressive growth of the unit fibrils of collagen after their initial formation

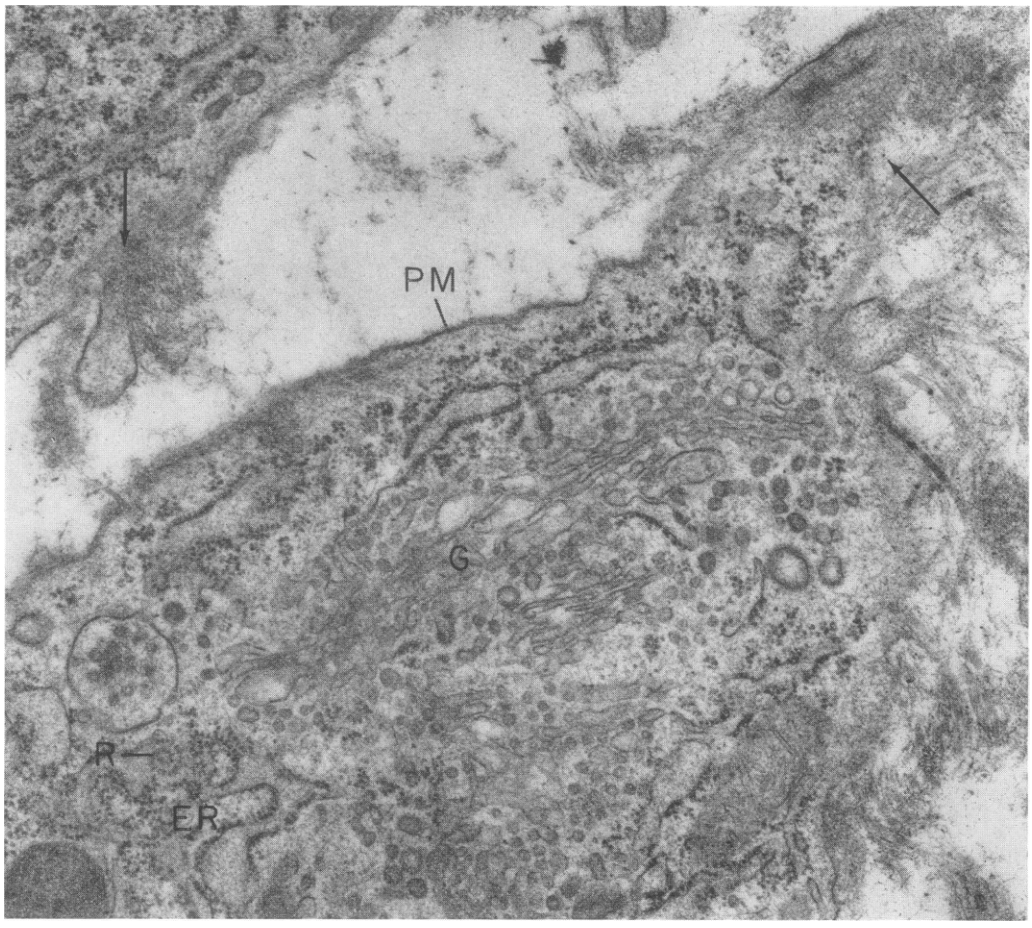


FIGURE 14 As in the image in Fig. 13, this represents part of a fibroblast from the stroma of a hepatoma. Here also the plasma membrane (*PM*) appears more or less distinct. At some points (as at arrows) the fibrous material of the cell cortex blends directly with extracellular material of the same texture. The Golgi (*G*) is prominent; the ER is represented by numerous particle-studded cisternae (*ER*); and particles are frequently in polysome aggregations as at (*R*). × 31,500.

has been repeatedly noted. Starting from a diameter of 250 A in the skin of a 12-day-old chick embryo, they increase four- to fivefold to achieve in the adult tissue a measure of 1,000 to 1,200 A. This is the maximum fibril size for chicken skin (8). It is a peculiar fact of collagen fibrils, as pointed out earlier, that they reach a certain diameter and then stop and that the magnitude of this maximum differs in different tissue locations. Early in the developing organism collagen is readily soluble in dilute salt solutions, but with aging of the animal it becomes progressively more difficult to extract requiring finally dilute acids to accomplish its mobiliza-

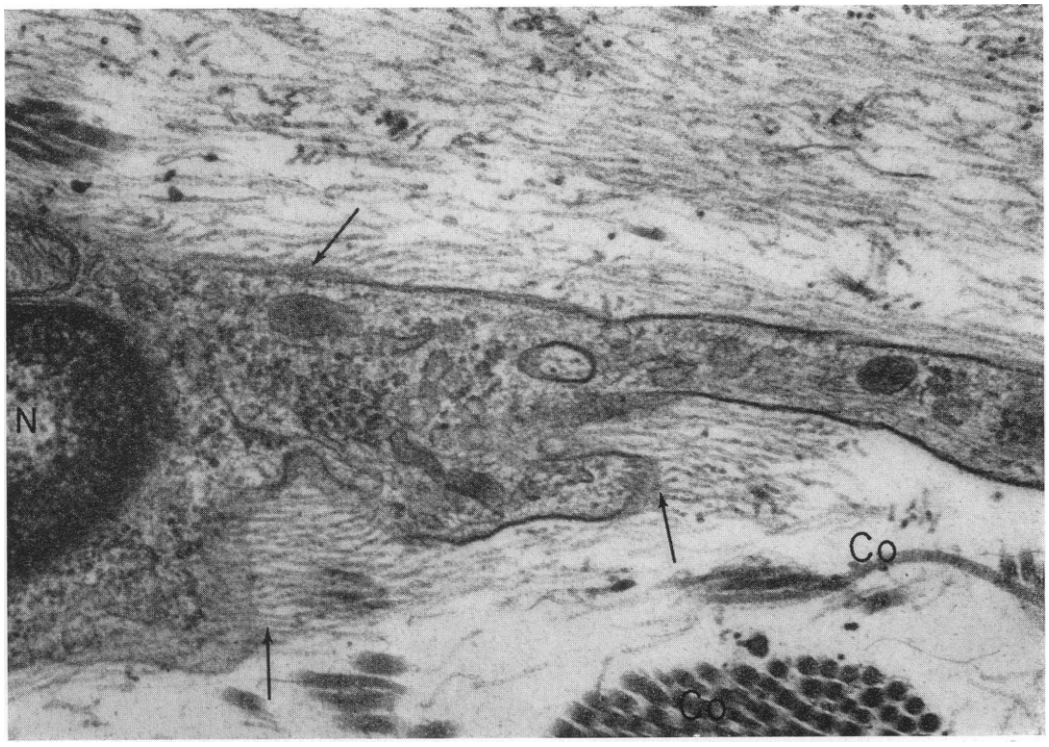


FIGURE 15 Part of a fibrocyte from the lamina propria of a bat's esophagus. The micrograph is especially valuable for showing the intimate association frequently displayed between fibrocyte surface and extremely fine, presumably protofibrils of collagen (arrows). The mature, fully formed collagen fibrils are shown at *Co*; the cell nucleus at (*N*). × 54,500.

tion (31). The obvious inference is that the initial or primary fibrils grow at the expense of tropocollagen in their environment until they reach a certain size at which point the forces attracting and holding further molecules are inoperative. Presumably these forces vary with the character of the primary fibril, which may not be collagen but a mucoprotein. This topic is, in any case, beyond the scope of this review.

It is possible with antiserum specific for soluble collagen to interfere with fibril development in cultures of fibroblasts (13). Instead of well formed fibrils, one obtains only strands of fine fibrous material and clumps of precipitated collagen. We may reasonably assume that the fibrous material is not antigenic collagen; but whether it represents the templates or primary fibrils for collagen deposition, unused under these antiserum conditions, remains to be investigated.

One of the most puzzling features of collagen deposition is the geometric ordering shown by fibrils or bundles of fibrils in the lamina propria and basement lamella of vertebrates. In the most common form, the fibrils are disposed in layers

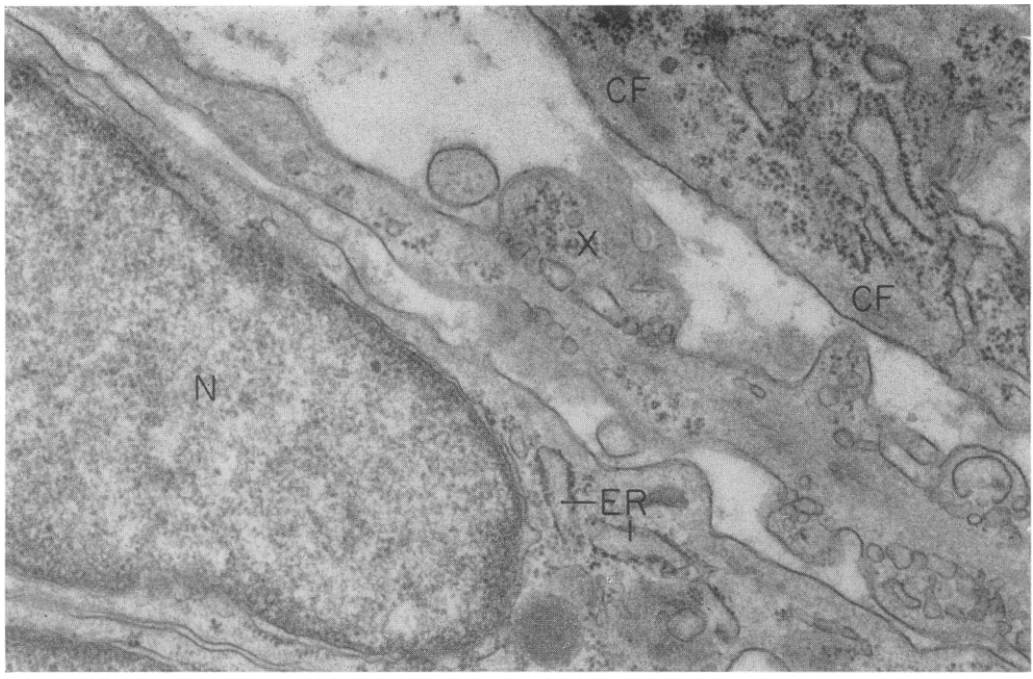


FIGURE 16 This micrograph includes parts of three fibroblasts from the stroma of a growing rat hepatoma. The nucleus of one is indicated at (*N*), cortical fibrous masses of another at (CF). Between these two cells there is a long slender pseudopodium of a third. It shows several surface blebs or excrescences of the type indicated at *X*. These are, in part, separated from the cytoplasm by a row of vesicles characteristically found in other situations associated with apocrine secretion. The vesicles in fusing would separate the bleb from the body of the parent cell (see text). × 33,000.

or plies with their long axes parallel in any one ply but running at right angles to one another in adjacent plies. This orthogonal grid arrangement is especially well displayed in the basement lamella of amphibian larvae, just under the epidermis (32, 33). Edds and Sweeny (34, 35) have studied this lamella *in extenso* and have collected information describing it as collagenous but have not reported definite clues to its morphogenesis.

The problem that interests everyone who studies this structure, and which is appropriate for this discussion, is how such an ordered structure is achieved if collagen formation is a completely extracellular phenomenon. With cell intervention one can imagine that a tissue of cells might imprint a design on the otherwise random arrangement of the first fibrils formed—an arrangement or ordering that would be maintained as additional units were added by fibroblast intervention. The most probable location for this guiding influence in basement lamella formation is in the epidermal cells, under which the ordering makes its first appearance

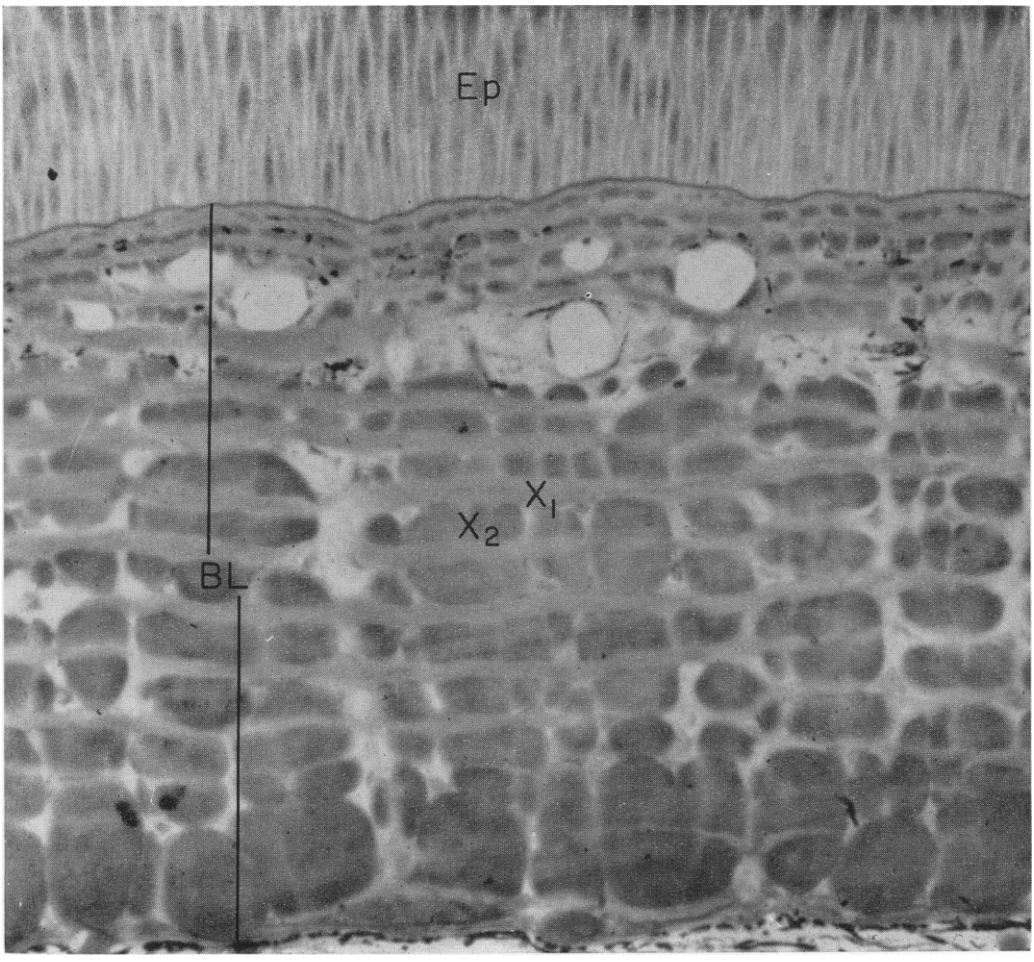
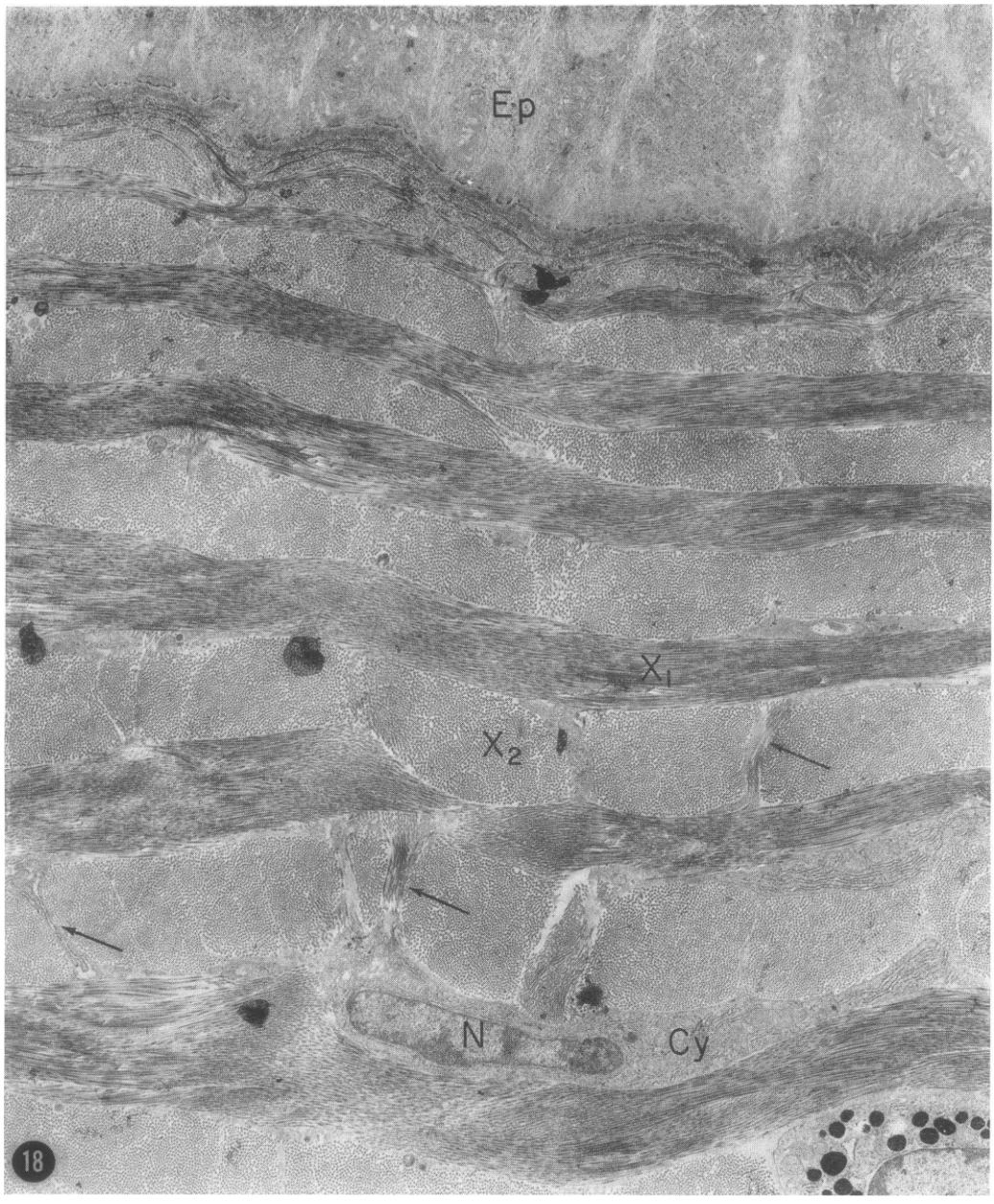


FIGURE 17 This is a light micrograph of a vertical section through the skin of a young adult lamprey. The epidermis (*Ep*) at the top is supported on a thick basement lamella (*BL*). This is constructed of bundles of collagen in orthogonal array. In successive layers, X_1 and X_2 for example, the orientation of the bundles shifts 90°. In X_2 and in alternate layers the bundles appear in cross-section; in X_1 in longitudinal section. It is evident that the bundles, especially those cut in cross-section, increase in size toward the innermost or basal surface of the lamella. Compare with Fig. 18 showing thin section from same piece of tissue. × 800.

(33, 34, 36). When labeled proline is introduced into a system in which the growth of a basement lamella is in progress, the label concentrates at the epidermal-lamella junction as though deposited there from the epidermis (36).

A vastly thicker basement lamella than that found in amphibian tadpoles may be observed in the skin of the adult lamprey. Even in a relatively small an-



imal there are 25 to 30 layers or plies in the lamella. The basal layers are made up of large bundles of collagen fibrils, but toward the epidermis the bundles are smaller and are less perfectly arranged in layers (Figs. 17 to 19). A few small bundles are oriented normal to the plane of the layers and the epi-

dermis (Figs. 18 and 19). The unit fibrils within the bundles of the bottom layers (presumably the oldest layers relative to the life of the animal) are uniform in diameter at 600 A. These obviously represent the mature fibrils. In the adepidermal zone, however, in which the fibrils are less numerous and bundles have not formed, the unit fibrils vary in size from extremely small ones of 50 A diameter up to the maximum size noted above. Close to the hyaline layer, under the basal surface of the epidermal cells, the fibrils are smallest (Fig. 19). On the basis of observed continuity between unit fibrils and this hyaline layer, one could propose that the fibrils take their origin in this layer. This layer would in turn seem to depend on the epidermal cells for its formation and structure. It may indeed be cut off from the basal surfaces of these cells, but our investigation of this system is as yet too preliminary to describe this event with certainty.

Of greater significance to the present review are some observations on the fibroblasts which invade the basal layers of the lamella. These cells, which have the structure of typical secreting fibroblasts, send small processes in between the mature collagen bundles and invade layers up to within the 7th or 8th from the epidermis. They could be making tropocollagen available for the development of new fibrils in these upper layers which are too far removed from the subcutaneous tissue to receive it by diffusion. In addition, these cell processes in many instances have finer fibrous units associated with their surfaces or immediately adjacent to them. The orientation of these fine units may parallel that of the mature fibrils nearby (Fig. 20), or they may be arrayed normal to them as in Fig. 21. Such small fibrils are never seen within the mature bundles, only at their peripheries. If they represent primary fibrils as is assumed, it would seem further that they are deposited and left behind in these locations by the resident fibroblasts. Thus these cells, sensitive as they are known to be to stresses, may detect the lines along which additional fibrils are needed and contribute the primary fibrils by ecdysis of their surfaces. It is interesting to note that in the mature fibrils a core of low density is uniformly present, suggesting that the central element is indeed of a different nature from collagen.

FIGURE 18 This is a low power electron micrograph of the adepidermal layers of the basement lamella in skin of adult lamprey. The epidermis (*Ep*) is shown in part at the top. These layers and the bundles of collagen fibrils which constitute them increase in size toward the basal surface of the lamella. In successive layers the fibrils change direction by 90°. At X_1 they lie in the plane of section; at X_2 they are normal to the section. Small connections between layers showing the same orientation are indicated by arrows. These connections include not only fibrils but also fibroblasts which from their fine structure would appear to be active in the production of collagen (see cell with nucleus (*N*) and cytoplasm (*Cy*) marked). Small parts of fibroblasts are included in the section at levels up to within a few layers from the epidermis. × 6,000.

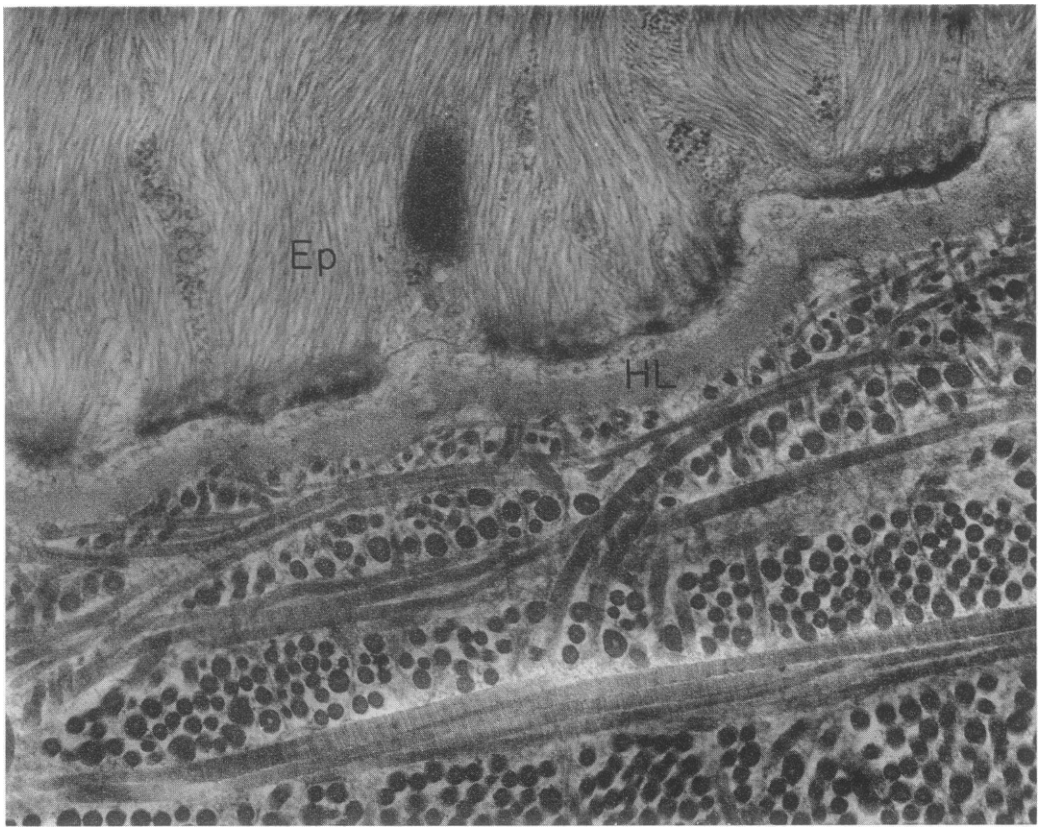


FIGURE 19 Micrograph showing junction between epidermis and basement lamella in young adult lamprey skin. The basal pole of an epidermal cell is shown at *EP*. The cytoplasm is packed with keratin filaments except for one or two regions containing ribosomes. Just beneath the basal cortex, which shows numerous densities representing half-desmosomes, there is a relatively clear zone and then the so called hyaline layer (*HL*). The collagen fibrils evident beneath the hyaline layer vary in size and show substantial randomness in their orientation. In deeper layers this latter feature is replaced by order. Cross-sections of the collagen fibrils are generally round and include a central spot of low density as though the core unit has not fixed or was originally different and has perhaps dissolved out. × 46,000.

It was noted just above that the unit fibrils of collagen in the uppermost or adepidermal layers of the basement lamella are continuous with a hyalin layer which coincides in position and appearance with the so called basement membrane underlying epithelia in general. Hyalin layers or basement membranes of this texture are indeed more widespread than is perhaps generally recognized. They are subjacent to Schwann cells of nerve fibers and morphologically continuous with the fibrils of the endoneurium. They also surround the fibers of smooth and striated muscle, and the collagen fibrils of the tendons are inserted in them. The

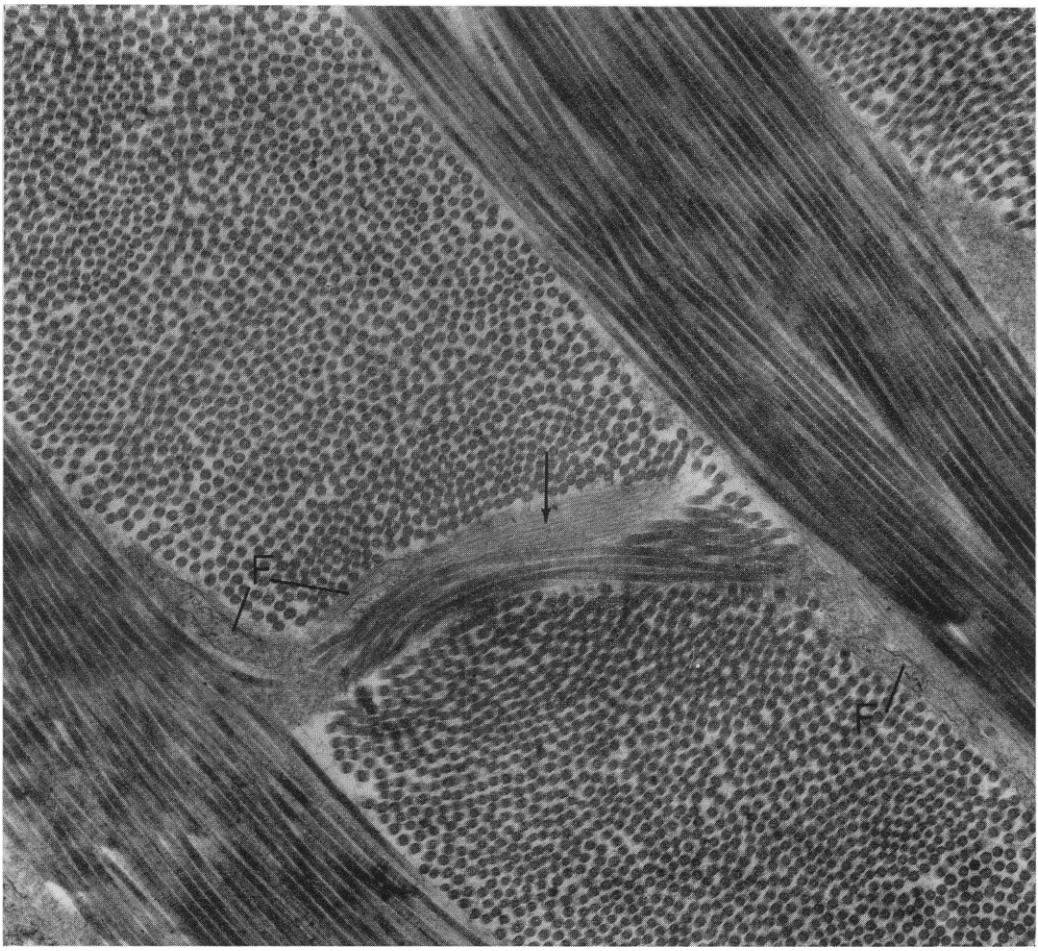


FIGURE 20 Micrograph of three deeper layers in basement lamella of lamprey skin. The features of particular interest are the cross-connection, the small cell processes associated with it (*F*), and the fine fibrils (arrows) presumably protofibrils of collagen lateral to the adjacent bundles of mature collagen. In this instance the protofibrils run parallel to the unit fibers of collagen in the adjacent bundles. In other instances the orientation is at 90° to the adjacent fibrils (Fig. 21). × 22,500.

layers of finely fibrous material adjacent to the surfaces of fibroblasts, and out of which collagen fibrils emerge in their morphogenesis, have the same texture as these widely occurring hyalin layers or basement membranes. These observations and others which might be cited in a more extensive treatise all describe an intimate relationship between collagen formation and these mucoprotein-rich layers adjacent to cell surfaces. The obvious implication is that the initiating element or elements in collagen fibril formation may reside in these layers in patterns which influence

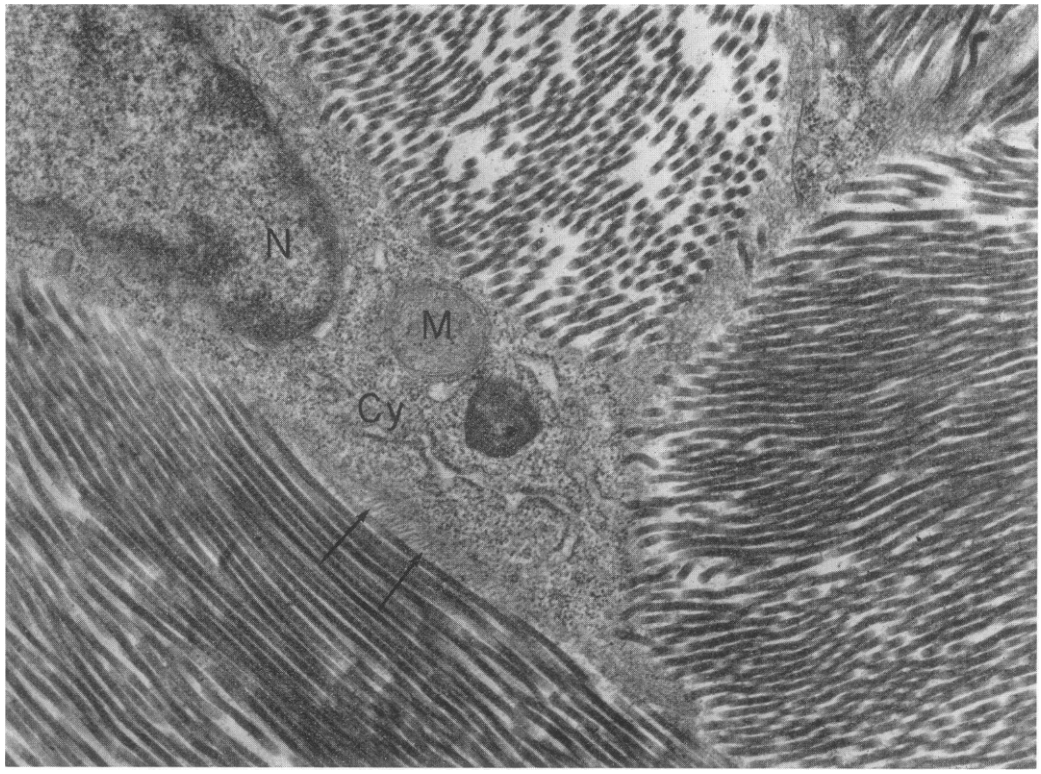


FIGURE 21 Micrograph similar to that in Fig. 20 except that it includes more of invading fibroblast, including its nucleus (*N*), and parts of ER-rich cytoplasm (*Cy*), and mitochondrion (*m*). The array of protofibrils at the arrows is on the cell surface and oriented at right angles to the unit fibers in the adjacent bundle. × 22,000.

the orientation of the fibrils in the collagen fabrics characteristic of different tissues. Basement membranes, produced by the cells they surround or underlie would, according to this succession of responsibilities, receive their information from the cell of origin.

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