Collagen Formation by Fibroblasts of the Chick Embryo Dermis

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ABSTRACT

This investigation has sought to determine the relation between collagen fiber and fibroblast during fibrogenesis. Toward this end the surfaces of chick fibroblasts grown under *in vitro* conditions have been examined with the electron microscope after fixation in OsO_4. Supplementary information has been obtained from thin sections of fibroblasts fixed *in situ* during phases of fiber production.

The evidence provided by these studies and by various conditions of the experiments indicates that the unit fibrils of collagen form in close association with the cell surface. They were never observed within the cell. When these unit fibrils form in bundles it appears as though templates of some nature, possibly coinciding with stress fibers within the cell cortex, influence the polymerization of the fibrils out of material available at the cell surface. From here the fibrils and bundles of them are shed into the intercellular spaces and there grow to limited diameters by accretion of materials from the general milieu.

INTRODUCTION

Although the origin and formation of collagen fibers has been studied and debated for over a century, reports of observations and conclusions, even up to the present time, are contradictory and inconclusive. As recently as 1954 Klemperer (15), drawing on years of observation and study in this field, concludes that "the relationship between cell and intercellular substance of the connective tissue. . . is just as controversial (a question) as it was" in the middle of the 19th century.

To review again the history of investigations into this controversial problem and its several ramifications seems unnecessary for the purposes of this report. Besides the brief but excellent treatment of the historical aspects in Klemperer's article, there is a summary in Cameron's Pathology of the Cell (5) and literature reviews in papers by M. Lewis (17), M. Stearns (29, 30), F. K. Studnicka (31), and F. Wassermann (33), to mention only a few. The broad problem is one of defining the role of the fibroblast in collagen production. A subdivision of this, which is more especially the concern of experiments reported here, is the site of initial fiber formation. Do the fibers form apart from the cell in the ground substance of the intercellular spaces as proposed by Maximow (19), Nageotte (21), Doljanski and Roulet (7); within the cell to be later shed, as M. Lewis (17) proposed years ago; or are they formed at the cell surface, which is to say at the interphase between cell cortex or ectoplasm and the extracellular ground substance (Studnicka (31))?

Interest in the question derives from a number of sources. We are, primarily, interested in the mechanisms involved in the biogenesis of extracellular coverings and matrices, of which collagen plus ground substance constitute an important example. Cuticular formations as found in the invertebrates is another, and cellulose walls of plant cells yet another. In each of these instances, complex fiber patterns are evident, and it seems difficult to divorce the cell and its genetic information from an intimate connection with fiber formation.

In recent years, since attention was focused by the studies of Highberger *et al.* (10) and of Vanamee and Porter (32) on fiber formation in the test tube from solutions of collagen (Nageotte (21)), a

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strong inclination has become evident to relegate control of *in vivo* fiber production to the inanimate, physicochemical conditions of the intercellular milieu. Thus Gross *et al.* (9) in a recent article suggest that the monomeric form of collagen "is synthesized in the cell and transformed into fibrils in the environment of the extracellular space." This view, we feel, neglects the fact that considerable organization is frequently evident in the disposition of collagen fibers, as for example in the dermis of the skin, the perineurium (20), and in the cornea (14). Such complex patterns, we believe, must depend on a preceding cellular design.

It, therefore, seems of some interest to report in full some studies on collagen-producing chick fibroblasts, which have engaged our interest over the past few years. The observations arising from these investigations have convinced us that unit fibrils of collagen in the dermis of the skin where some order is evident, are produced initially at the cell surface and thereafter shed by the cells into the intercellular ground substance. Here they may individually increase in size, and "mature."

Materials and Methods

Collagen-producing cells (fibroblasts) from chick embryo skin have provided the material for these studies. In the preparation of *cultures*, the explants were taken from the skin over the back and neck of 8- and 9-day-old embryos. The epidermis was not separated from the dermis in preparing the explants except in a few instances when it was done with the intention of observing whether its presence or absence made any difference in the production of collagen. Tissue for *thin sectioning* was taken directly from embryos ranging in age from 9 to 16 days of incubation, although the greatest attention was given to 12- and 14-day-old specimens, since these were considered as approximately equivalent in stage of development with cultures of 9-day-old skin kept for 3 to 5 days *in vitro*. Sections were also cut from adult chick skin to determine the diameter of mature unit fibrils.

Cultures for the most part were prepared on flying coverglasses over Maximow well-slides. In the earliest experiments of this study, the cultures were grown in nutrient-rich medium containing plasma, embryo extract, and chick serum. The results were briefly described by Porter and Vanamee in 1949 (27). In the later studies, from which this report is drawn, cultures were grown in a much simpler medium consisting of Locke's solution plus 10 per cent bouillon.

The culture procedure used was as follows: The skin from the dorsal surface of the embryo was removed with iridectomy scissors and placed in Locke's solution at pH 7.2. Here the tissue was cut up into small pieces which were thereafter rinsed in an additional bath of Locke's solution. Next, the explants were transferred (4 or 5 to a culture) to coverglasses (22 x 22 mm.) which had been previously coated with formvar and sterilized following procedures described elsewhere (24). The excess of Locke's solution was withdrawn and replaced with a shallow drop of Locke-bouillon culture medium. The cultures thus prepared were affixed to Maximow slides and incubated as lying drops for the first 24 hours, and then inverted as hanging-drop cultures so that loose tissue debris would fall away from the formvar-coated surface and not appear as contaminants in the final preparations.

At selected intervals and/or when a suitable population of fibroblasts had appeared in the cultures they were harvested for microscopy. Preparatory to fixation the cultures were rinsed in two changes of Earle's balanced salt solution, pH 7.4. While in each rinse, a pipette was used to gently flush the culture. This was done to dislodge any loose collagen fibers which might otherwise settle out on the cell surfaces during drying. While still bathed in a shallow drop of balanced salt solution, the culture was inverted over 0.05 ml. of OsO_4 (2 per cent in H_2O) in a well-slide for 10 to 20 minutes. Following this, it was washed in H_2O and marginal areas of the culture were transferred to uncoated electron microscope grids, as described earlier (24). When the preparations had dried they were lightly shadowed with metallic chromium (at an angle of 12°) to enhance the contrast of the fine fibrils on the surface of cells or formvar support.

When light microscope preparations were required, the cultures were grown in the same manner on uncoated coverglasses, fixed with neutral formalin, and silver impregnated according to Foot's modification of Bielschowsky's procedure, and finally stained with Van Giesen's.

For reasons that will be clarified in the following pages, it was important to obtain thin sections of the cultured cells. The procedures employed for obtaining these were essentially the same as those described recently by Borysko (3), and by Howatson (11). Since, however, the cultured cells, and more especially their exposed surfaces, were destroyed or badly damaged by these procedures, recourse had to be taken to the examination of fibroblast in whole tissues.

The majority of thin sections studied were, therefore, derived from small pieces of the embryo skin fixed *in situ*; with buffered OsO_4. It was found necessary for the successful preservation of this tissue to have the fixative at pH 8.0, and to have present in the solution a small concentration of calcium ions. The calcium ($CaCl_2$) was added to the fixative just before use, to give a final concentration of 0.001 per cent. Fixation was continued for ½ hour, after which time the fixed material was dehydrated in the usual manner and finally embedded in methacrylate. Sections were ultimately cut

at 50 to 100 mμ and examined without removing the plastic.

Sections were also prepared from the same embryos for light microscopy. The tissue was fixed in neutral formalin and embedded in paraffin. Sections were cut at 5 μ, then impregnated with silver (Gomori's modification of Bielschowsky's procedure), and stained with hematoxylin and eosin.

OBSERVATIONS

A careful study of mats of collagen produced by fibroblasts *in vitro*, reveals that each unit fibril, or fiber bundle, is confined to a single level or plane in the mat and does not weave from one level to another (Fig. 1). It was recognized, as pointed out earlier (23), that this lamellar organization placed in question the concept of collagen formation as a free polymerization of fibers from *in vivo* solutions of molecular collagen. Fiber development of this latter type is exemplified in clot formation from solutions of fibrinogen, where the individual fibers weave in a random fashion through the clot (26). The fact that the collagen fibers in these *in vitro*-formed mats do not show this type of disposition suggests that collagen fibrogenesis is not a random process but one in which the cells probably operate in some manner to give the forming collagen fibers the organization demonstrated. In cultures, this type of organization would be achieved if the cells produced fibers in successive waves of activity and/or deposited the fibers in migration over the existing mat. In any case, some form of intimate association between cell and fiber seems unavoidable, and it appears that the cell is involved not only in producing the fiber, but also in its initial orientation. By way of examining this concept of fiber formation it was decided to grow fibroblasts in as simple a culture medium as possible so that there would be no source of collagen or collagen-forming materials other than the tissue explants and the cells derived from them. As mentioned above, an earlier study by Lewis (17) indicated that a medium consisting solely of Locke's solution and bouillon was adequate for the development of fibers from chick embryo skin. Therefore, in these present experiments the Lewis procedure was duplicated in all esential features, the single departure being the use of beef bouillon rather than chicken.

Relation of Culture Age to Fiber Production.—In most instances cultures were grown from explants of skin from 8- or 9-day-old chick embryos. After 24 hours of incubation the explants are surrounded by a margin, or halo, of fibroblasts adherent to the coated surface of the coverglass. During the next 48 to 72 hours, migration of fibroblasts proceeds at a fairly active pace and seems, under these circumstances, to reach a limit somewhere between 72 and 96 hours of incubation. At this time fiber development is much in evidence and it appears that the fibroblasts have reached that stage in their differentiation at which they most actively produce collagen. From this point on for 9 or 10 days, the cells survive and continue to produce fibers if the medium is changed every 48 hours. These changes in the living culture and especially in collagen production are readily observed with the light microscope, using polarized light illumination, or in fixed preparations stained with silver (Figs. 2 and 3).

With the higher resolution of the electron microscope it is possible to identify the fibers formed as collagen and to follow them from the earliest stages of their development. Preparations for such a study were obtained by harvesting the cells at intervals of 24, 48, 72, and 96 hours after the cultures were set up.

As expected, after 24 to 30 hours of growth and cell migration there was scant evidence of fiber development. In general, the cell surfaces were smooth and there was little evidence of fibers of the stress (or fibroglial) type in the cytoplasm of the migrating cells. Cells of this type were shown in an earlier publication (23).

After 48 hours the picture presented by the fibroblasts was considerably changed. The smooth cell surface and the cytoplasm now showed fibrous thickenings or condensations. Some of these were continuous with fine fibrils extending across the intercellular spaces while others appeared simply as short fibrous condensations at the cell sruface. It is apparent in cultures of this age that fiber production is already in progress.

The progression toward cell differentiation and active functioning was shown more strikingly by the cells in the 72-hour-old cultures. It is assumed that these cells are producing collagen at about the same rate as fibroblasts *in situ* in the skin of the 12-day-old chick embryo. The cultured cells show prominent bundles of unit fibrils coursing across their surfaces (and in many cases passing from cell to cell) plus a large number of independent unit fibrils (Fig. 4).

These differences with culture age are shown in more exaggerated form in preparations of cells from cultures 96 hours old and older. In fact, in

cultures 5 to 6 days old, the whole surfaces of the fibroblasts seem to be contributing to fiber production (Fig. 19).

Distribution of Fibers in Single Cultures.—If we assume that prior to fiber development the contributing molecular collagen is in solution in the culture medium, and that the cells play no intimate role in fiber formation and orientation, then it is reasonable to expect to find fibers distributed more or less uniformly over the cells and cell-free spaces within the limits of the drop of medium. Under this assumption, the cells and original explant would produce the building blocks of collagen fibers, these would diffuse away from the cells into the medium (Locke's-bouillon), and when these are present in sufficient concentration, fibers would begin to form. Such an assumed sequence of events in fiber formation would be in keeping with the extracellular theories of Maximow (19), Levi-Montalcini and Sacerdote (16), and others.

A number of observations which have a bearing on the above assumptions have been made on the following *in vitro* preparations.

Cultures, after 96 hours' incubation, were examined with the electron microscope in order to observe the distribution of fibers relative to the individual cell colony. The preparations used in this study were made from colonies of cells grown from groups of five explants, arranged in a circle on a single coverslip. The drop of medium covering the cluster of explants was shallow at the outside margin, and deeper between the explants.

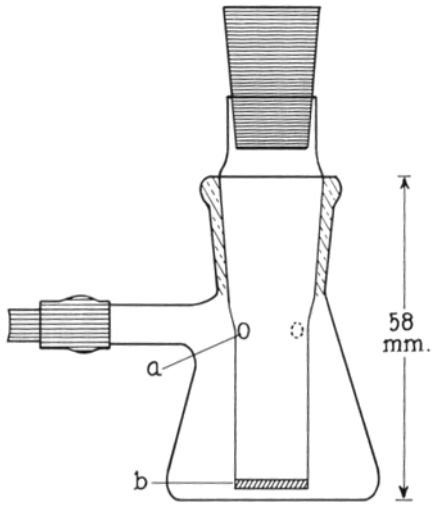
It is evident from the examination of these preparations that the distribution of fibers is not uniform over the whole culture, but bears a definite relationship to the density of the cell population in the individual colonies. In other words, beyond the margin of the colony where there are no cells, there are no fibers, although culture medium was present, and it is only at the margin of the colony where there are a few cells, that the fibers begin to appear (Fig. 5). The cells here are relatively undifferentiated in terms of fiber production; the cell surface shows only early stages of condensations preceding fiber appearance. As the examination moves in toward the explant, an intermediate zone may be identified in which there are still spaces between the cells, and fibers are present in considerably greater abundance (Fig. 6). There are two kinds of fibers: single unit fibrils which course about at random, and bundles (called collagen fibers) made up of fine unit fibrils which streak across the field of the micrograph in more or less straight lines and generally along radii from the center of the cell colony. Finally, in the next zone, arbitrarily defined as that area where the cells fill in the available space and overlap to some extent, there is an abundance of fibers (Fig. 7). Here the independent (single) unit fibrils are more numerous and the compound collagen fibers (fibril bundles) are larger and clearly more advanced in development than in the other zone.

In summary, then, the number and development of collagen fibers in a colony parallels the distribution of the cells. Beyond the margin of the cell colony, even where there is abundant medium, there are few or no fibers. The same gradation in fiber frequency is evident in silver-stained preparations examined by light microscopy (Fig. 2).

Gross Fiber-Cell Relationships.—A second observation of significance in regard to the origin of the collagen fibers has to do with fiber-cell relationships rather than with fiber-colony relationships. It has been noted in these and earlier preparations that the fiber bundles passing over a cell surface are in parallel array and frequently fairly evenly spaced (Figs. 9 and 10). On numerous occasions such fiber arrays have been observed to continue on over an adjacent cell and sometimes over several cells where these latter form a more or less continuous sheet. It is also noteworthy that in many instances the direction adopted by these fibers is normal to an axis of polarity which passes through the centrosome and nucleus (Figs. 4 and 10). It would seem that wherever the cells are freed from the stresses and distortions of migration they slow down and form a loose sheet, and that under these circumstances the fiber bundles which form, bear a definite relation to axes of the cells. It is difficult to imagine how such fiber-cell relationships could be the result of anything but an intimate association of cell and fiber during fibrogenesis.

Finally, in defining the site of fiber formation, cognizance must be taken of the fact that fibers appear in close association with the cells even in hanging drop cultures. Under such culture conditions, fibers forming away from the cells would be expected to settle out into the apex of the hanging drop and from there be washed away when the culture is rinsed in Earle's solution preparatory to fixation. Actually, this does not happen, as indicated by the abundance of fibers over and between the cells even after repeated washings.

Absence of Collagen-Forming Materials in Culture Medium.—It is possible to devise a number of experi-



TEXT-FIG. 1. Outline drawing of filter-culture flask. The inner glass tube, which is fitted into the outside flask with a ground glass joint, represents the culture vessel. A 10 mm. diameter Selas porcelain filter disc (1/10th inch thick and No. 02 porosity) closes the bottom end of this vessel at *b*. In the experiment, explants of tissue and cells were grown in the inner surface of this filter. Two holes marked *a* allow for equalization of pressure and gas mixtures in the two air spaces. The side arm, here closed with a rubber sleeve, was used for gassing the cultures without disturbing the inner glass tube.

ments to test for the presence of a freely diffusible fiber-forming material in the media of these cultures. Doljanski and Roulet (7), in studies of the same problem, arranged their cultures on a sintered glass filter in a manner that would allow collagen in solution to pass through and form fibers on the acellular side of the filter. They reported that fibers did indeed develop without benefit of intimate contact with the cells. In order to repeat this crucial experiment we devised a setup similar to Doljanski's (Text-fig. 1). Explants of chick skin were placed on the upper side of a porcelain filter disc, and were allowed to grow for 6 days. At the end of this time the fluid on the side of the filter away from the cultures was examined for fibers with the electron microscope. The results were uniformly negative—fibers were never found. We were, therefore, not able to confirm Doljanski's observations under the experimental conditions employed.

In another attempt to demonstrate fiber-forming material in the medium surrounding the cultured cells, a series of cultures of chick embryo skin was prepared in flasks and incubated for 6 to 7 days. At the end of this time the supernatant was removed and clarified by centrifugation. No fibers were detected when the sediment from this centrifugation was examined in the electron microscope. The supernatant from the centrifugation was then mixed with NaCl to give a final concentration of 1 per cent. If collagen were present it should, on the basis of previous experience, precipitate out under these conditions (32), but again the results were negative.

From these various observations it would appear that fiber-forming material, in detectable quantities, is not present in the culture fluids. One may conclude that fiber formation takes place chiefly at the cell surfaces from materials which are present there, or that fiber formation uses up available material as fast as it is released from the cells.

Fiber-Cell Relationship During Fiber Formation.—The problem here is to define the exact relationship of the fiber to the cell during fibrogenesis. Do the fibers form just within the cell surface or do they polymerize out of material at or on the cell surface? The answer to these questions depends on a visualization of the plasma membrane and since this is difficult to accomplish with cultured cells, part of the observations bearing on this problem must be derived from thin sections. Initially, however, something was learned about the process of fiber formation by simply studying the surfaces of fibroblasts during their differentiation.

In the first place, it appears that fibroblasts in the process of fiber formation have a material of mucinous nature on their surfaces, for strands of mucinous material frequently appear to be pulled off the surface. Such strands, or fibers, may extend from cell to cell, as in Fig. 8, or from cell to substrate. They have the appearance of having been left behind as the cell withdraws from a former position. They occur commonly among cells that are actively migrating at the time of fixation, as, for example, at the margin of the cell colony. When newly formed, as one may assume those in Fig. 8 have been, the fibers give no evidence of periodicity. The final disposition of this particular type of fiber is in doubt, but since fibers without striae have not been encountered in older cultures it is concluded that these strands either organize in time into typical collagen fibers or go into solution Among cells located more centrally in the colonies, fibers of this type are rarely if ever encountered. This may reflect a lesser degree of migratory activity or it may be related to the fact that the centrally situated cells are further along in their differentiation. If this is a method of collagen fiber formation it is by far the least common. However, it demonstrates, perhaps better than any other observation, that there exists an amorphous material at the cell surface.

In most cells, especially in cultures equivalent in age to 10- to 12-day chick embryos, it is common to find material at the cell surface forming long ridges. These ridges may be oriented more or less in one direction with respect to the cell, and could conceivably coincide with stress (or fibroglial) fibers. In some instances, these ridges, or thickenings, extend over the whole length or breadth of the cell and are organized in parallel arrays (noted also above) (Fig. 10). The width of these thickenings varies greatly. Some are less than 50 mμ in diameter; others may be 200 to 300 mμ. The margins are ill defined and appear to blend with the general cell surface. The margins are also irregular, and branching from them is frequently encountered. It is evident from such images that a material, or substance, has concentrated along lines (possibly of stress) at the cell surface or in the cell cortex. In many instances, especially in cells of 48-hour-old cultures, the material of these ridges is quite amorphous, and there is no evidence of a fibrous component. However, this would appear to be only a temporary phase, for in most instances, especially in cells of 72-hour-old cultures these longitudinal thickenings show a content of fine fibrils. The fibrils are almost completely hidden in some cases (Figs. 11, 13, and 14) but in others they stand out with considerable prominence and show the clear cut periodicity of collagen (Fig. 15). The broader of these fiber-forming ridges may contain large numbers of fine fibrils constituting a bundle (Fig. 11) whereas the narrower ridges may show only one or two fibrils (Figs. 11 and 15). One is led to conclude that the larger, essentially straight bundles of fibrils noted here and elsewhere in these cultures have their origin in these longitudinal thickenings at the cell surface.

It frequently happens that fibroblasts in culture show tapered ends and the fusiform configuration characteristic of these cells. In the electron microscope image the two margins of the tapered ends are usually more dense than the rest of the cell, and have the general appearance of the longitudinal thickenings described above (Fig. 12). Frequently they show fine fibrils embedded in this thickened material and in some instances the fibrils show a periodicity. Such thickenings as these are encountered along other cell margins as well (Figs. 12 and 13).

Fiber bundles that appear to have their origin at the cell surface and that are oriented along lines of stress coinciding with radii from the colony center, are the most prominent elements in the total collagen production. It is these bundles that are visible in the light microscope after silver impregnation. In what follows, these structures will be referred to as the oriented fibers or fiber bundles.

Besides these bundles, there are always evident fairly large numbers of essentially unoriented fibrils in any field of cells and collagen fibers. These are present usually as unit fibrils, singly or in groups of two's or three's. They course about among the fiber bundles in the form of broad curves and are without apparent orientation, unless, as sometimes happens, they join a larger bundle or fuse with other independent fibers to form a small compound fiber bundle. They constitute a distinct class of fiber as far as these preparations are concerned and also apparently in certain details of their formation.

Again in this regard, reference must be made to cells in cultures of 48- to 72-hour incubation where early fiber formation is in progress. The surfaces of these cells sometimes show ridges of material in the form of arcs and occasionally complete circles. In low power micrographs, as in Fig. 16, they give the impression of having developed through the pulling back of surface material forming the arcs or whorls. Thus the surface of a cell may be covered by these arc-shaped ridges which define the outlines or margins of these withdrawal areas (Fig. 16). Where such ridges are numerous they run into one another and because of their rounded shape appear to be continuous (Fig. 16). Out of such thickened areas (contiguous whorls) fibrils appear to polymerize (Figs. 17 and 18) much as they did out of the straight (stress) ridges discussed above. The curved, unoriented form of the ridges and the fibrils arising from them are preserved in the form of the unit fibril after they have left the cell surface.

As a general rule, then, a material at the cell surface migrates into, or by some unknown forces is drawn into longitudinal concentrations which ultimately become sites of fiber formation. This process shows a number of variants, perhaps related to the conditions of *in vitro* culture. It does not seem essential, for example, that the fiber forming substances concentrate in longitudinal ridges for fiber development to take place. In a few instances very complex networks of fine fibrils have been found apparently emerging from the cell surface. It seems, in some instances, as though a thickened layer of surface material may transform into a sheet of fibrous elements. It may be that these organize into bundles at a later time, but at their inception nothing of the sort is evident.

In the 5- to 8-day-old cultures, when the rate of fiber production has greatly accelerated, fairly dense mats of collagen fibers are found adjacent to the fibroblasts. The cell boundaries seem less definite, and almost the entire surface of these cells appears to be transforming into collagen (Fig. 19). This and other aspects of fiber production continue until the 9th or 10th day, beyond which time the cells will not normally survive under these conditions of culturing.

It is technically feasible, and of considerable interest, to search the cell surface for evidence of the very earliest organizations of this surface material into fibrils. Unfortunately, the complex and irregular nature of the surface, after fixation and drying, does not provide a good background for the visualization of the macromolecular unit. Hence, images of such units are not available. It is, however, possible to get micrographs of early condensations of fiber-forming materials, and close study of such images shows some longitudinal organization of an extremely fine fibrous material which is presumably a reflection of the form of closely packed protofibrils or early unit fibrils of collagen (Fig. 20).

Cell-Fiber Relationship in Situ from Thin Sections.—From the topographic images of cultured cells it is obviously difficult to decide on the precise relationship of the fiber to the cell, *i.e.*, whether the fiber forms within the cell membrane or outside it. Sectioned material seemed more likely to supply the required information. Thus a study of thin sections of cultured fibroblasts and of chick embryo skin was undertaken. Cultured fibroblasts proved to be extremely fragile under the procedures of fixation and embedding employed; hence only fragmentary observations were possible in sections of cultures. These were sufficient, however, to give a picture of the fiber-cell relationships and to convince us that they repeat those evident in the whole skin. In the case of the latter material, observations have been confined to the part of the dermis just beneath the basement membrane. Here the cells are flattened in a plane parallel to the epidermis but otherwise are of irregular contour. Sections thin enough for electron microscopy, cut from an object as polymorphic and irregular in shape as fibroblasts, show cell fragments in all imaginable aspects. The chance of having a section coincide precisely with the longitudinal axis of a forming fiber is quite remote. In spite of this, however, sectioned material proved to be valuable for studies supplementing the observations on whole cultured cells.

The sections of skin from 8-day-old embryo showed no significant evidence of fiber formation. At 10 days, however, small numbers of fiber bundles can be discerned in the same tissue and by 12 days these are prominent. The individual or unit fibrils present at this time are extraordinarily fine, measuring approximately 250 A in diameter. The events in fibrogenesis taking place *in situ* in a 12-day-old embryo can be assumed to coincide roughly with those occurring in a 72- to 96-hour-old culture from 9-day-old explants. Fibrogenesis is probably progressing at a much greater rate in 14-day embryos. In any case, by this time the concentration of intercellular fibers is much greater than at 12 days. It was this very evident difference in fiber development that made the small sample represented by sections of 14- and 16-day-old embryo skin the more useful for studies of fiber-cell relationship. The abundance of fibrous material present in the skin of this age embryo (skin taken from midline dorsal) is easily demonstrated by silver staining procedures (Fig. 21). The region just beneath the epidermis is especially heavily stained. This suggests a greater concentration of collagen fibers in this region although it is to be recognized that it may simply reflect a particular architecture of cells and fibers which serves to hold more fully the deposition of silver. Regardless of the relationship between this dense band and collagen, it is evident that the standard silver procedure indicates the presence of abundant collagen. This observation is clearly borne out by the electron micrographs of thin sections through the same region of skin from the same embryo.

In Fig. 22 it is possible to recognize beneath the basement membrane (*bm*) with its special structure (see figure legends), a collection of fiber bundles and pseudopodia of fiberblasts. The cells in this region are flattened in planes parallel to the basement membrane, and have numerous and well extended pseudopodia. In sections these latter appear as small segments of cytoplasm between the fiber bundles and are identified by their content of mitochondria or other cytoplasmic elements. The fiber bundles in this particular figure appear either in cross- or longitudinal section, which means that they lie roughly at right angles at successive levels in the dermis. The intervening layers of cells constitute a tissue whose function is assumed to be that of forming this pattern of fibers. The unit fibrils of the bundles are of uniform diameter along the length shown. The variation in diameter from fibril to fibril is mainly due to differences in the

plane of sectioning. Each fibril shows the characteristic periodicity of collagen. The bundles vary greatly in the number of unit fibrils contained, as well as in shape. The smaller, more flattened bundles are closer to the basement membrane; the large bundles, having as many as 75 or more unit fibrils always are deeper in the dermis. In Fg. 22 the few profiles of cell surfaces depicted are not shown at sufficient magnification to reveal details of fiber-cell relationships even where such relationships are displayed. Considerably more of the nature of fibroblast structure is shown in Fig. 23. This is a somewhat oblique section including portions of three cells with a number of fiber bundles between them. The cytoplasm of these fibroblasts is characterized by the presence of profiles of a large number of elongate elements of the endoplasmic reticulum. These are bounded by a membrane which owes its density in part to a large accumulation of small dense granules (22) on its outer or matrix surface. These membrane-limited elements plus granules constitute the ergastoplasm of these cells. The profiles have been shown to represent transverse sections through flattened vesicles or cisternae rather than longitudinal sections through tubules. These vesicles in fibroblasts have a content that is more dense after osmium fixation than is the surrounding matrix of the cytoplasm. Such an abundance of reticular elements with associated granules, is characteristic of cells engaged in protein synthesis, and supports the view that these fibroblasts are actively synthesizing a product for fiber production and growth. Other components of the cytoplasm are identified as Golgi elements, mitochondria, and fibrous matrix. The latter is evident in the cell which passes from lower left to upper right of the micrograph as the homogeneous regions from which other elements of the cytoplasm are excluded, and probably represents a more highly gelated part of the cytoplasm. In the original micrographs these regions appear to contain a condensation of an extremely fine fibrous material.

Where the plane of sections is normal to the cell surface, this surface is defined (except where it is disrupted) by a dense line which represents the plasma membrane. At places along this membrane there are occasional regions of increased density intimately associated with extracellular bundles of fibrils. An examination of these dense areas frequently reveals the presence of points of greater density comparable in size to the cross-sections of fibrils which lie further away from the cell surface. It is difficult to avoid the impression that these points of density at the plasma membrane and just within the cortex represent the earliest polymerization of materials into fibrils. We assume that cross-sections through the fiber bundle in Fig. 15 would appear as at *A* in Figs. 24 and 25. There are a number of places in Fig. 24 where bundles of fibrils in cross-sections are associated with regions of cortical densities (see also at *B* and *C*); another is shown in Fig. 26. In the same or adjacent fields there are a number of instances where extracellular fiber bundles are close to the cell surface without any noticeable change being evident in the cell membrane and cortex. The former with associated cortical densities seem, therefore, to be special regions from which fibers are being contributed to a bundle already in formation. This contribution may be pictured as a form of shedding from the cell cortex, *i.e.*, ecdysis.

Just as there are extracellular fiber bundles without associated cell-cortex densities, there are extracellular densities without prominent bundles of fibrils. In some cases, as at *A* in Fig. 23, outside the cell surface there is a mass of material which is amorphous but which also shows evidence of fibrous elements. Associations of relatively unorganized dense material, outside and inside the level of the plasma membrane (Figs. 28 and 28*a*), probably represent sections through such essentially fibrous condensations as shown in Fig. 20. In a few instance such associations have appeared without evidence of any membrane between them. The presence of a matrix material, essentially amorphous at regions of fiber formation, is especially well depicted in Fig. 27. This shows a grazing section cut to give what may be described as a sliver off the cell surface. The plasma membrane is shown at *pm*, a thin layer of unorganized material at *c*, and leading out of it at a more exterior level relative to the membrane is a parallel array of fine fibrils showing evidence of collagen periodicity. These are taken to represent early unit fibrils of collagen. It is worth noting that fibrils a little further removed from the cell membrane, but included in this section, run at right angles to the finer ones embedded in the surface matrix. They are also large and presumably represent fibrils developed at an earlier age in the differentiation of this tissue. This same right angle disposition of fibers in the subepidermal region of the skin is evident in other figures, *e.g.*, Fig. 22.

Extracellular Growth of Fibers.—It was early apparent that unit fibrils forming at the cell surface

possibly from a template resident there were more slender than the units in the dense mats found in older cultures. This fact suggested that (*a*) either slender unit fibrils had been fusing laterally to form larger fibers, or (*b*) each unit fibril had grown in diameter by simple accretion of unpolymerized "monomeric" collagen from the medium. If the former possibility operates, we should encounter evidence of branching; thus far, however, none has been seen. Instead, the unit fibrils in a bundle run parallel over considerable distances and in some cases show a tendency to retain a uniform distance from their neighbors. Also, if the larger fibers were formed by lateral association of smaller fibrils, we should expect to see evidence of their compound structure in cross-section, but none has been observed. Therefore the second possibility seems the more likely.

Evidence of unit fiber growth with increase in age of the fiber deposits is not difficult to obtain. A simple comparison of the size of unit fibrils in cultures after 4 days of incubation with similar units developed *in vitro* over 8 days shows a striking difference in fiber diameters. In the younger cultures they measure about 300 A; in the older ones, about 600 A. As can be seen in Figs. 29 and 30 and also in Fig. 1, there is no evidence of branching. It seems to follow, therefore, that increase in size has resulted from a gradual growth (presumably by accretion) of the earliest unit fibril.

This same development may be observed by comparing fiber size in comparable regions of skins taken from 12-day-old, and 18-day-old embryos and adult chickens. In the early embryo the fibrils measure between 200 and 300 A (Fig. 31). Their equivalent in the 18-day-old embryo is about 800 A (Fig. 32). And in the adult, similar fibers range from 1000 to 1200 A (Fig. 33). In cross-section the larger fibers are homogeneous and appear as units. The cross-banding expected in collagen fibers is evident in fibers from animals of all ages; the spacing varies from 450 to 500 A with evidence of intraperiod banding.[1]

DISCUSSION

Site of Collagen Morphogenesis.—The investigations described above have sought to define the site of origin of collagen fibers and the sequences in their development. It has become clear from the various procedures and approaches used that unit fibrils of collagen form in close association with the cell surface. Expressed in other words, it appears that in the material studied, these unit fibrils organize out of or polymerize from material that is at the cell surface, and from here, the fibrils, in many cases already in bundles, are shed into the intercellular spaces. There is clear evidence that after being shed, the unit fibrils expand in diameter (without loss of density) by accretion of materials from the general milieu of the intercellular spaces.

In support of the above conclusions we propose to discuss certain of the points of evidence.

Relative to the site of origin, it should be necessary only to point to the micrographs of the cell surface with associated fibers to be convinced that this is where they form. There are, however, certain other explanations which might be given for this relationship and which might sound plausible if not examined. It might, for example, be said that after fixation and during drying preparatory to examination, fibers in the medium in various stages of formation simply settle out or dry down on the cell surface. This could be accepted as an explanation for their position if certain facts were not in evidence. Of first importance among these is the observed relation of fiber to cell. By this is not meant soley the intimate blending of fiber with cell surface and the emergence of the striated form from the apparently unorganized material at the surface, but also the grosser aspects of fiber organization with respect to cells. For example, any "drying-down" of erstwhile floating fibers would be expected to result in a random distribution of fiber directions. At least they would be expected to show kinks and bends resulting from the various forces exerted by the migration of a phase boundary across the cell surfaces during drying. In actual fact, however, it is not uncommon to find the fibers in nearly parallel array and in some instances almost equally spaced in these arrays. Also it has been noted that, especially in relation to fibroblasts that have organized into a more or less continuous sheet, the fibers continue across cell boundaries over points of contact between the cells, and occasionally along radii from the center of the cell colony. It is to be noted in this connection that Dueggeli (8), in an experimental application of tensile stress to cultures of subcutaneous rabbit tissue, found that fibers developing near the explant are arranged parallel to the line of pull. This

[1] In a separate study made by one of us (Pappas) it has been found that methacrylate embedding induces shrinkage in collagen fibers and a consequent shortening of the normal 640 A spacing.

arrangement of fibers reflects an intimate relation between cell and fiber and suggests that the fibroglial or stress fiber, developed initially in the ectoplasm of the fibroblast, gives direction to the subsequent formation of extracellular fibers (6). A direct transformation of one into the other has not, however, been demonstrated.

Further support for the intimacy of fiber and cell during fibril development is found in the fact that the fibrils are not easily dislodged from cells during preparation procedures. In actual fact the surface of the culture can be vigorously flushed with balanced salt solution without noticeably disturbing the fibers. If they are simply floating in the medium, or even if they were bound together into a loose framework, effects of washing and drying should be in greater evidence. Finally, one may reasonably point to the fact that the concentration of fibers is directly proportional to the concentration of cells. In other words, fibers are few at the colony margin and very dense at the colony center. This is true in spite of the existence of a continuum over the cells in the nature of culture medium in which collagen molecules could diffuse freely and yield, upon polymerization, fibers uniformly distributed within and beyond the margin of a cell colony.

This association of fiber with cell is not surprising if it is recalled that in most tissues the bundles of collagen fibers are oriented with respect to some dimension of the tissue or tissue components. In the sections of chick skin, *e.g.*, it was observed that the fiber bundles among the fibroblasts are oriented at right angles at different levels in the tissue. This doubtless is achieved by a previous organization of the cells in those areas, which in turn reflects the pattern of development. The same "organization" of collagen fibers is evident in a wide variety of tissue, *e.g.* tendon and perineurium (20), with the highest degree of orientation found in the cornea (14).

Specific Relationship of Fiber to Cell Surface.— With the above point established, the question that presents itself is that of the specific or precise relationship of the fiber and cell during morphogenesis. Does the unit fibril, or bundle of such fibrils, organize from the viscous material which, in some instances at least, coats these cells, or do the fibrils form beneath the cell membrane to be later shed? The picture obtained from the surfaces of the cultured cell relates only the impression that the fibrils organize within a condensation or ridge of dense amorphous material. It is not evident from such micrographs where the plasma membrane is with respect to the fibril bundle and its matrix. One would gain the impression, in some instances from the flatness of the cell surface over these longitudinal densities, that the membrane covers the area. Sections of cells forming fibers are of obvious importance in settling this problem. When taken from tissues which are identical with those used in culturing they show in selected cases what seems to be an interruption of the plasma membrane. Thus in cross-section, the more immature fibrils are surrounded by matrix material just at the level of the membrane, whereas the more mature and sharply defined fibrils are outside this level. It would seem correct, therefore, to speak of the precise site of formation as at the cell surface rather than on or beneath the surface.

Some of the surface densities apparent in sections of cells do not show differentiated fibers of 250 A diameter, and may in fact show little or no evidence of a fibrous component. These are thought to represent sections through such surface condensations (fibril-free) as shown on the cultured cells pictured in Figs. 5, 9, or 11.

The fibrils which form in these regions of the cell surface are usually 200 to 300 A in diameter. It is possible that they have a fairly uniform diameter at the time of differentiation but that the nature of the preparation, the relation of fibril to section, or other factors prevent one from getting an exact measure of their size.

Cross-sections are likely to be the more reliable and measurements taken of these are indeed quite uniform at this stage in fiber formation. After leaving the cell the fibril gains in girth. There is no evidence to indicate that this comes about through association of smaller fibers to form larger fibers. Instead, the individual fibril would appear to grow through the addition to its surface of monomeric collagen from the intercellular milieu. Recently, Gross *et al.* (9) have been able to extract a soluble collagen from young calf corium. The authors suggest that this collagen exists in the ground substance in monomeric form. The process of gradual growth through surface accretion is distinguished from the differentiation of the original unit fibril. The latter apparently represents the core upon which subsequent layers of "collagen" are deposited.

These observations can be related in several respects to problems of collagen fibrogenesis that have been investigated and argued for several decades. They easliy confirm, for example, the

conclusions of M. Lewis (17) (subsequently supported by observations of Stearns (29, 30) and others) that fibrin plays no direct role in collagen production. If, as seems justified, one agrees that fibers having a 640 A periodicity are collagen, then collagen has formed in the present experiments in the complete absence of fibrin. The Lewis demonstration of the same was not universally accepted because not all workers could agree that the fibers which formed in her cultures were collagen. There now seems to be little excuse for such doubts and not only this but several other conclusions derived from the investigations of M. Lewis deserve to be credited with a high degree of accuracy.

It appears to follow also, and in agreement with many earlier workers, that the fibroblast is indispensable for fiber formation. This was concluded by Lewis (17), Maximow (19), Doljanski and Roulet (7), Levi-Montalcini and Sacordote (16) and numerous others. It was stressed and beautifully demonstrated by Stearns in her studies of the sequences in wound repair as observed in the transparent chamber of the rabbit's ear. Fiber formation occurred behind, and never in front of the advancing margin of migratory fibroblasts. The same conclusion is supported here not only by the evident close physical association between cell and fiber but also by the complete absence of fibers on the substrate beyond the margin of the culture.

The precise relation of cell to fiber in fibrogenesis, also widely debated over the years and not settled to everyone's satisfaction even now, seems to gain some clarification from the above observations. The view early expressed by Boll (2) and thereafter subscribed to by Mall (18), Lewis (17), Studnicka (31), and others that the primary fibrils form at the cell surface, possibly from materials available in the ectoplasm (cortex) of the cell finds strong support. On the other hand, no fibers were noted at definite intracellular locations. It is true that electron micrographs of whole cells do not permit one, in some instances, to define the precise location of a fiber with respect to the cell surface, but images derived from thin sections can be interpreted more accurately and thus far no fibers bearing any resemblance to collagen have been seen within the cytoplasm. This is in sharp conflict with earlier reports of Jackson (12), who in micrographs of tendon cells noted the intracellular location of a sizable bundle of fibers. A more searching interpretation of the picture might have described the bundle as enwrapped by a cell but not contained within its cytoplasm.

On the other hand, no evidence was obtained from the current investigation for the development of fibers in extracellular locations. Since the more telling arguments against this view presented earlier by Maximow (19), Baitsell (1), and others, were derived from the culture experiments, it must be admitted that the unusual environment of the culture setup may have discouraged fibril formation except at the cell surface. We are not, therefore, prepared to deny that under some conditions encountered *in vivo*, fibers may form outside of and some distance from cells. That such may happen, especially in certain pathological conditions, has been maintained, but it is still to be doubted whether in such instances fibroblasts have not preceded in location the site of fiber development.

With extra- and intracellular sites placed in doubt, one is inclined to attach greater significance to the evidence defining the cell surface as the site of fibrogenesis. This is a general opinion supported by electron microscope studies of Wassermann (33) and by the more recent observations of Jackson (13). It conflicts, however, with the conclusions of Stearns, who finds fibers formed in association with fibroblasts or fragments of fibroblasts but places the exact site as extracellular. (Other statements within the same report conflict with her conclusions and make one wonder if observations on atypical cases had not been assigned undue importance.) The present study defines the ultimate association that may exist (and seems the rule in this material) between forming fibril and cell. The same conclusion seems to us to derive from the highly complex and regular patterns the fibers often show in the mature tissues such as the cornea (14)—patterns which, it seems, must derive from a preceding cellular framework.

Viewed as described in this paper, fiber production by fibroblasts seems to parallel quite closely the production of fibrous sheaths of cells in general. Reed and Rudall (28) have shown that the cuticle of the earthworm must obviously come from the cells of the underlying epidermis. Electron microscope studies show it to be composed of tight bundles of fibrous units and as these are traced to the cell surface the impression is obtained that they are formed at the surface and thence shed to the environment. Indeed, Bradbury and Meek (4) have recently shown a sheath-like covering on the surface of fibroblasts in the leech. Embedded in the matrix of this sheath are parallel arrays of collagen fibers. Evidence of ecdysis from

cells of higher animals is not available except possibly for the basement membrane of the amphibian epidermis. Here vertical sections through the skin show what may be interpreted as successive stages in fiber development, and material for this development seems to be organized and contributed by the basal cells of the epidermis (25, 34). The story for collagen development appears to be very much the same, except that the material for fiber production is condensed into ridges which may coincide with lines of stress placed upon the fibroblast by conditions of its environment.

It develops from these various observations and considerations that two sites within the system studied are able to initiate and extend the polymerization of collagen. They may both be regarded as templates. The one, to be thought of as the primary site of formation, is at the cell surface, and, as mentioned just above, may coincide with stress fibers within the cell cortex. The other is the partly formed unit fibril, which grows to a characteristic diameter at the expense of monomeric collagen in the medium. The origin of the monomeric form is not settled, but if one is guided by relationships apparent in many other cell types between the endoplasmic reticulum and protein synthesis, one would suggest that the extensive development of this system in fibroblasts laying down collagen, indicates an active role in collagen synthesis. Presumably, from the cisternae of this system the monomeric form is discharged to the cell's environment and quickly induced to polymerize by enzymes resident in templates at the cell surface or in unit fibrils.

Bibliography

1. Baitsell, G. A., The origin and structure of a fibrous tissue which appears in living cultures of adult frog tissues, *J. Exp. Med.*, 1915, **21,** 455.
2. Boll, F., Untersuchungen über den Bau und die Entwickelung der Gewebe. IV. Die Entwickelung des fibrillären Bindegewebes, *Arch. mikr. Anat.*, 1872, **8,** 28.
3. Borysko, E., Recent developments in methacrylate embedding. II. Methods for the sectioning of optically selected single cells, the orientation of the plane of sectioning, and the identification of the region of the specimen included in the sections, *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** No. 4, suppl., 215.
4. Bradbury, S., and Meek, G. A., A study of fibrogenesis in the leech, *Hirudo medicinalis, Quart. J. Micr. Sc.*, 1958, **99,** 143–148.
5. Cameron, G. R., Pathology of the Cell, London, Oliver and Boyd, 1952.
6. Costero, I., Pomerat, C. M., Barroso-Moguel, R., and Chévez, A. Z., Tumors of the human nervous system in tissue culture. II. An analysis of fibroblastic activity in meningiomas, *J. Nat. Cancer Inst.*, 1955, **15,** 1341.
7. Doljanski, L., and Roulet, F., Studien über die Entstehung der Bindegewebs-fibrille, *Virchow's Arch. path. Anat. Physiol.*, 1933, **291,** 260.
8. Dueggeli, O., Über den gestaltenden Einfluss von Zugspannungen auf Bindegewebskulturen, *Z. Zellforsch.*, 1937, **26,** 351.
9. Gross, J., Highberger, J. H., and Schmitt, F. O., Extraction of collagen from connective tissue by neutral salt solutions, *Proc. Nat. Acad. Sc.*, 1955, **41,** 1.
10. Highberger, J. H., Gross, J., and Schmitt, F. O., The interaction of mucoprotein with soluble collagen; an electron microscope study, *Proc. Nat. Acad. Sc.*, 1951, **37,** 286.
11. Howatson, A. F., and Almeida, J. D., A method for the study of cultured cells by thin sectioning and electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 115.
12. Jackson, S. Fitton, The formation of connective and skeletal tissues, *Proc. Roy. Soc. London, Series B*, 1954, **142,** 536.
13. Jackson, S. Fitton, The fine structure of developing bone in the embryonic fowl, *Proc. Roy. Soc. London, Series B*, 1957, **146,** 270.
14. Jakus, M. A., Studies on the cornea: I. The fine structure of the rat cornea, *Am. J. Ophth.*, 1954, **38,** 40.
15. Klemperer, P., The significance of the intermediate substances of the connective tissue in human disease, *The Harvey Lectures*, 1955, **49,** 100.
16. Levi-Montalcini, R., and Sacerdote, E., I caratteri e le modalità di suiluppo delle fibre collagene e reticolari nelle colture in vitro, *Arch. ital. anat. e embriol.*, 1938, **40,** 37.
17. Lewis, M. R., Development of connective-tissue fibers in tissue cultures of chick embryos, *Carnegie Institution of Washington, Pub. No. 226, Contrib. Embryol.*, 1917, **6,** 45.
18. Mall, F. P., On the development of the connective tissues from the connective-tissue syncytium, *Am. J. Anat.*, 1902, **1,** 329.
19. Maximow, A., Development of argyrophile and collagenous fibers in tissue culture, *Proc. Soc. Exp. Biol. and Med.*, 1925, **25,** 439.
20. Maximow, A., Bindegewebe und blutbildende Gewebe, *in* von Mollendorff's Handbuch der mikroskopischen Anatomie des Menschen, 1927, **2,** pt. 1.
21. Nageotte, J., Sur le caillot artificial collagène, signification, morphologie générale et technique, *Compt. rend. Soc. biol.*, 1927, **96,** 172.
22. Palade, G. E., The endoplasmic reticulum, *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** No. 4, suppl., 85.

23. Porter, K. R., Repair processes in connective tissues, *Tr. 2nd Conf. Connective Tissues*, Josiah Macy, Jr. Foundation, New York, 1952, 126.
24. Porter, K. R., Observations on a submicroscopic basophilic component of cytoplasm, *J. Exp. Med.*, 1953, **97,** 727.
25. Porter, K. R., Observations on the fine structure of animal epidermis, *Proc. Internat. Conf. Electron Microscopy*, London, July 1954, 539.
26. Porter, K. R., and Hawn, C. von Z., Sequences in the formation of clots from purified bovine fibrinogen and thrombin: a study with the electron microscope, *J. Exp. Med.*, 1949, **90,** 225.
27. Porter, K. R., and Vanamee, P., Observations on the formation of connective tissue fibers, *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 513.
28. Reed, R., and Rudall, K. M., Electron microscope studies on the structure of earthworm cuticles, *Biochim. et Biophysica Acta*, 1948, **2,** 7.
29. Stearns, M. L., Studies on the development of connective tissue in transparent chambers in the rabbit's ear. I, *Am. J. Anat.*, 1940, **66,** 133.
30. Stearns, M. L., Studies on the development of connective tissue in transparent chambers in the rabbit's ear. II, *Am. J. Anat.*, 1940, **67,** 55.
31. Studnicka, F. K., Die Entwicklung der Bindegewebsfibrillen (Desmofibrillen). Zugleich ein Kapitel aus der Geschichte der Histologie, *Ergebn. Anat.*, 1944, **34,** 369.
32. Vanamee, P., and Porter, K. R., Observations with the electron microscope on the solvation and reconstitution of collagen, *J. Exp. Med.*, 1951, **94,** 255.
33. Wassermann, F., The intercellular components of connective tissue: origin, structure and interrelationship of fibers and ground substance, *Ergebn. Anat. u. Entwcklngsgesch*, 1956, **35,** 241.
34. Weiss, P., and Ferris, W., Electron-microscope study of the texture of the basement membrane of larval amphibian skin, *Proc. Nat. Acad. Sc.*, 1954, **40,** 528.

Explanation of Plates

Plate 65

Fig. 1. Electron micrograph of a dense mat of collagen fibers found in a 6-day-old culture of chick fibroblasts grown in a Locke-bouillon medium. Unit fibrils are seen to occur singly or in bundles forming the reticulin fiber of the light microscope image (Figs. 2 and 3). In the great majority of instances the fibrils and bundles are confined to one plane in the fiber mat and do not weave up and down through the depth of the mat. The unit fibrils show a periodicity of 220 A; the characteristic 640 A period is in slight evidence at point indicated by arrow. The material was fixed with OsO_4 vapors for 10 minutes and thereafter shadowed. × 37,000.

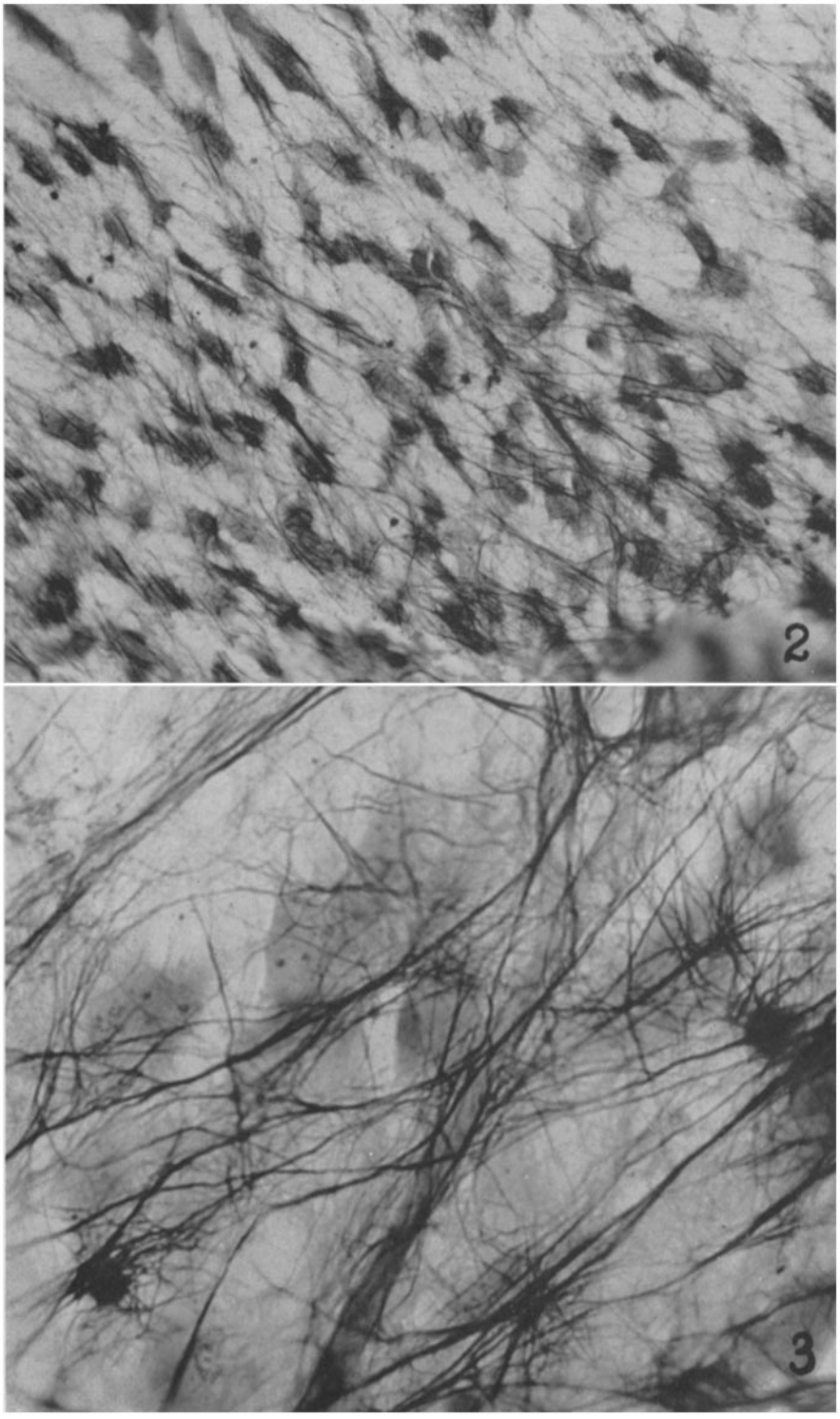
(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 66

FIGS. 2 and 3. Photomicrographs of cultures derived from explants of 9-day chick embryo skin, grown for 4 days in Locke-bouillon medium. The cultures were fixed with neutral formalin, impregnated with silver (Foot's modification of Bielschowsky's), and stained according to Van Gieson's procedure.

In Fig. 2 the explant can be seen in the lower right corner. Silver-stained fibers appear in greater numbers close to the explant, where the population of migrated cells is the most dense. × 300.

Fig. 3 is a higher power picture of the same culture showing silver-impregnated fibers and the fiber-fibroblast relationship. × 1700.



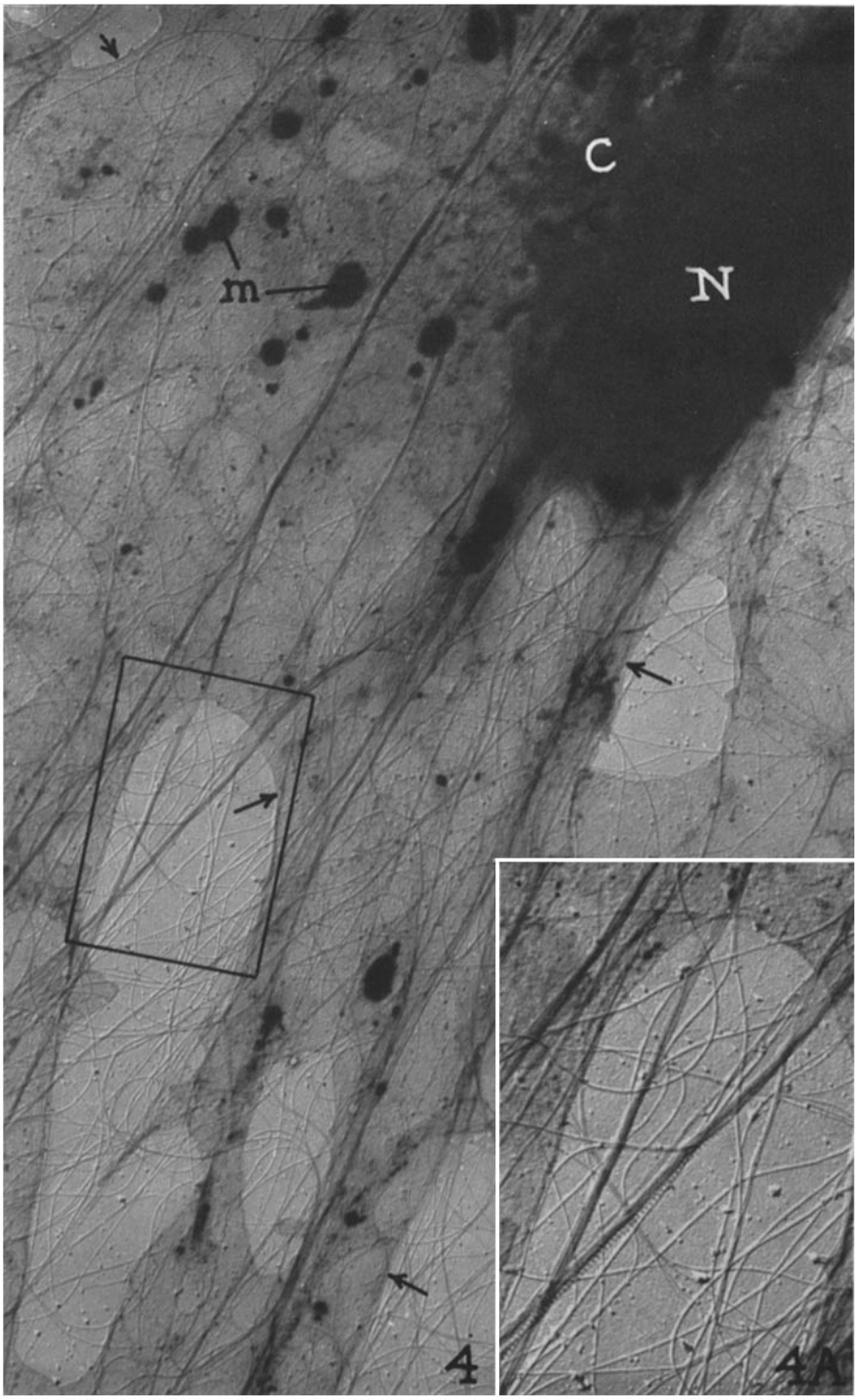
(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 67

FIG. 4. Electron micrograph of a portion of a fibroblast from a 72-hour-old culture of a skin explant from a 9-day chick embryo. The cells were fixed for 10 minutes with vapors from 2 per cent water solution of OsO_4. The preparation is shadowed.

The nucleus of the cell is indicated at *N*, the centrosome area at *C*, and cytoplasmic granules, presumably mitochondria, at *m*. Arrows point to various portions of the cell margin included in the micrograph, and the lower one points to the margin of an adjacent cell. Collagen fibers and unit fibrils are evident over the cell and over the extracellular regions included in the field. For the most part they are oriented parallel to the longer axis of the cell in the general direction of upper right to lower left. Departures from this are more common among unit fibrils than among bundles, *i.e.* collagen fibers. × 5500.

FIG. 4 *a* represents an enlargement of the area inside the rectangle in Fig. 4 and shows that the fiber bundles possess the characteristic periodic structure of collagen. × 10,500.

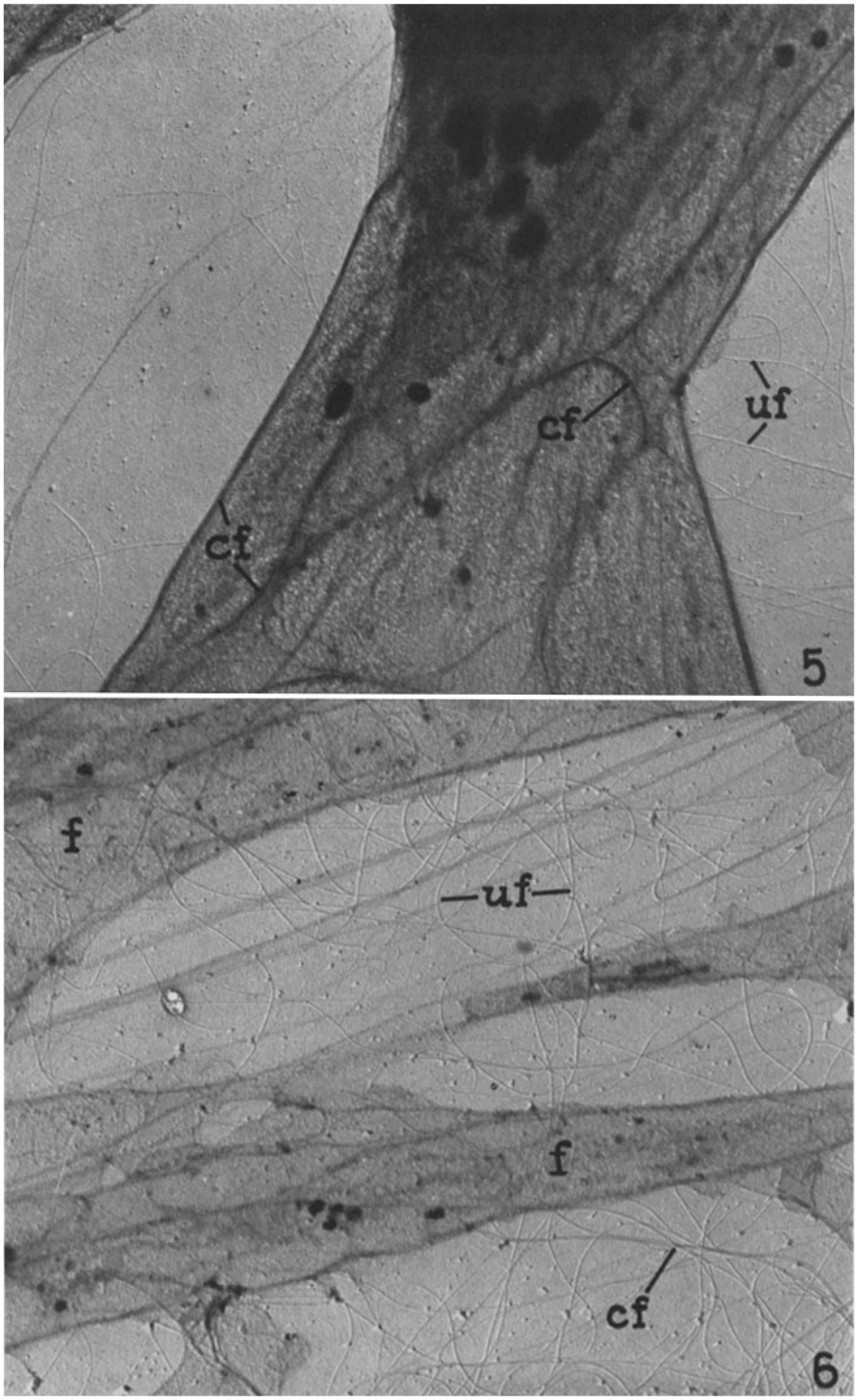


(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 68

FIG. 5. Electron micrograph of part of a fibroblast from a 96-hour-old culture of a skin explant from a 9-day chick embryo. This cell was selected from the margin of the colony where the cell population was sparse. Only a few unit fibrils (*uf*) of collagen are present in this area beyond the cell margins. What are interpreted as bundles of unit fibrils, forming collagen fibers (*cf*), are evident on the cell surface. Fixed over vapors of OSO_4 for 10 minutes and shadowed with chromium. × 6000.

FIG. 6. Electron micrograph of portions of fibroblasts (*f*) from the same culture from which the marginal cell illustrated in Fig. 5 was taken. In this case the area pictured was located closer to the explant. Spaces between the cells show a definitely greater abundance of collagen fibers. Unit fibrils are indicated at *uf*, and bundles at *cf*; both types are evident on the fibroblast surfaces (*f*) as well as between the cells. × 6000.

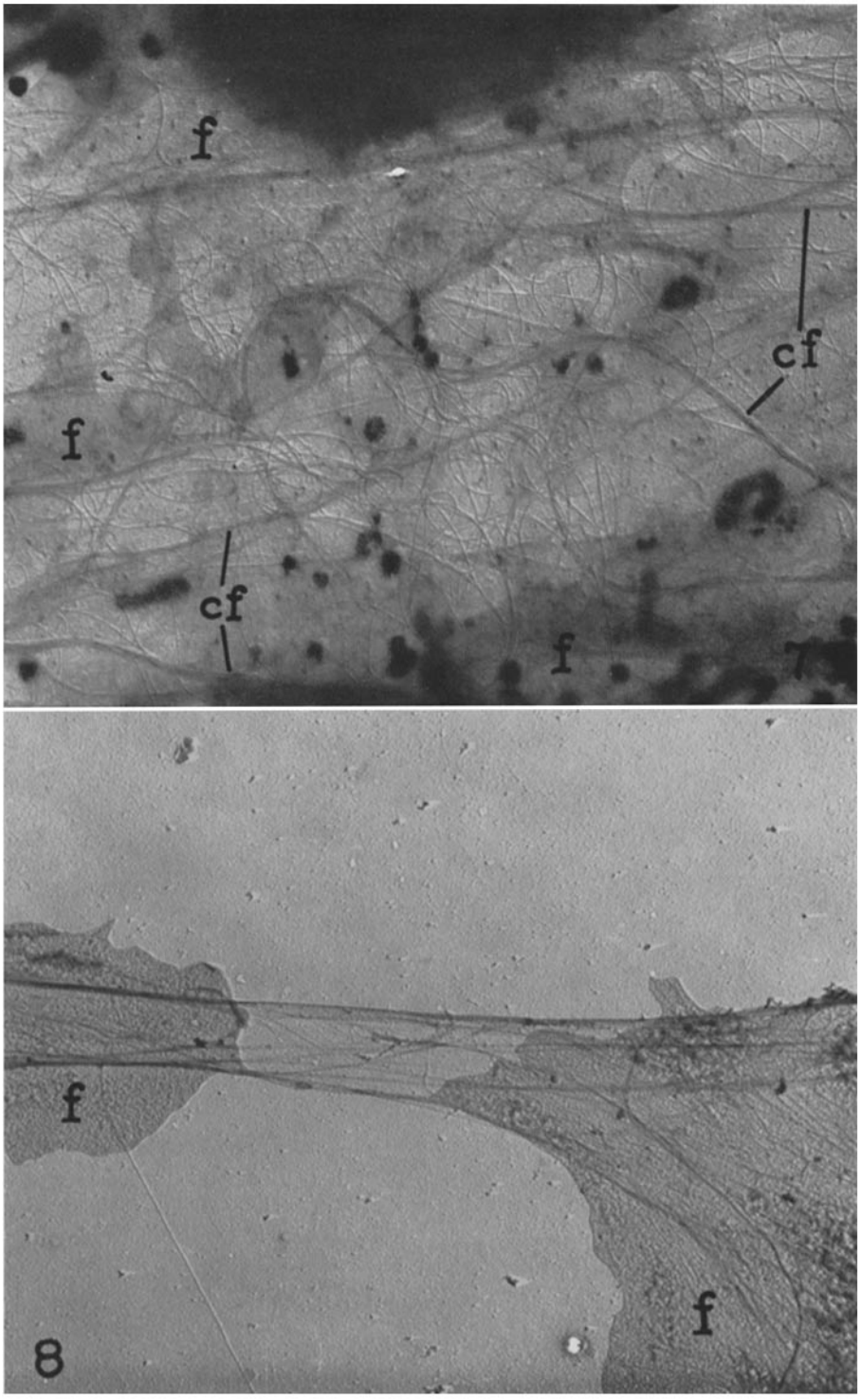


(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 69

FIG. 7. Electron micrograph of an inner area of the same cell colony shown in Figs. 5 and 6. This inner area—where the cells fill in almost all the available space and begin to overlap—has the greatest concentration of collagen fibers (*cf*). Portions of fibroblasts with mitochondria, etc., included are indicated at *f*. × 6000.

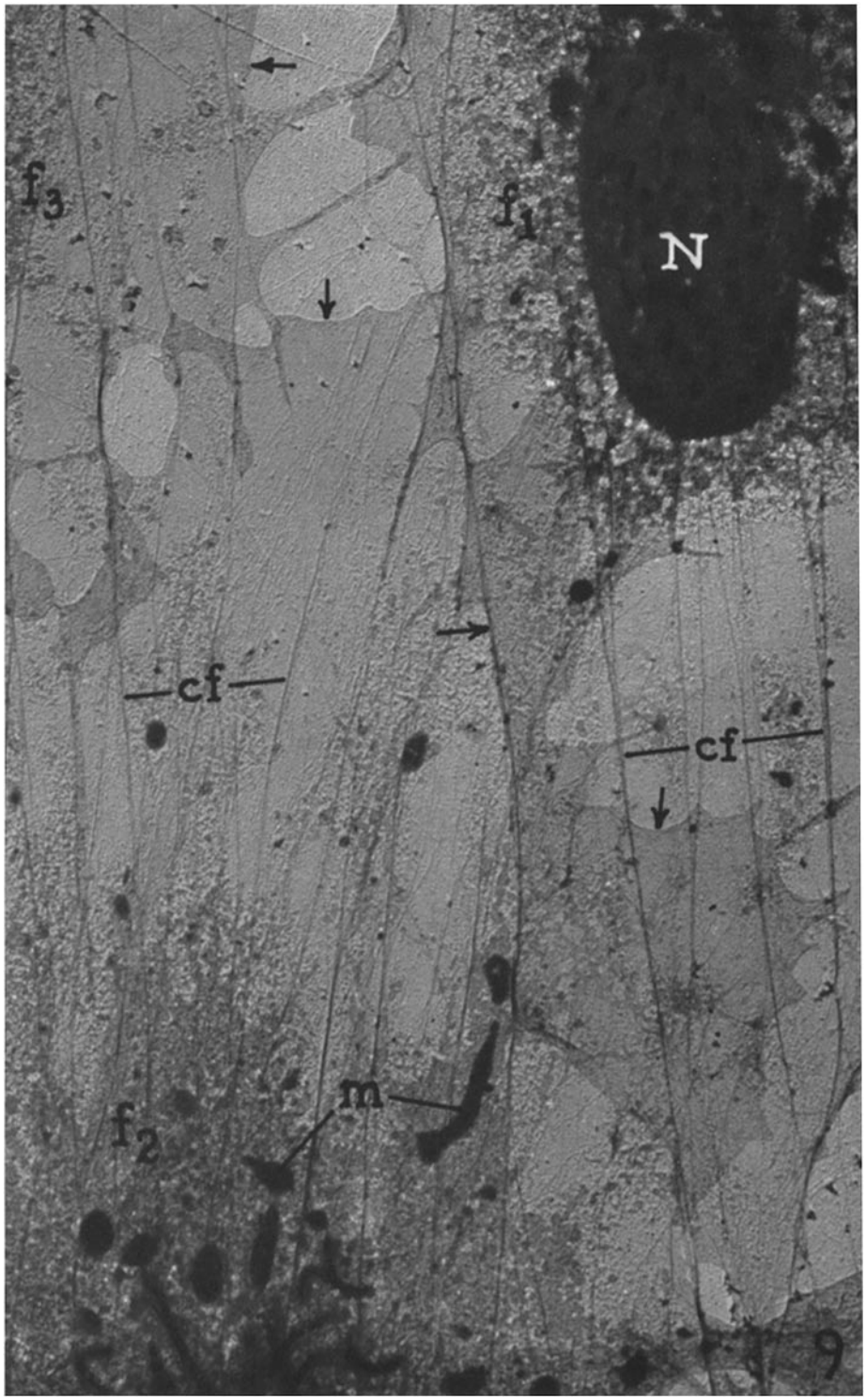
FIG. 8. Electron micrograph showing small portions of two fibroblasts from a 72-hour culture of skin from a 9-day-old chick embryo. The amorphous material on the surface of these two fibroblasts (*f*) appears to have been pulled out into fibers extending between the cells. These fibers are without striae. The cells are fixed with OsO_4 vapors for 15 minutes and shadowed with chromium. × 7000.



(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 70

FIG. 9. Electron micrograph of parts of three fibroblasts (f_2, f_1, f_3) from a 96-hour culture of an explant of skin from a 9-day chick embryo. A nucleus (N), mitochondrion (m), and collagen fibers (cf) are readily identified. The arrangement of parallel fibers on the fibroblast surfaces is continuous from cell to cell. Cell margins are indicated by scattered arrows. × 5000.

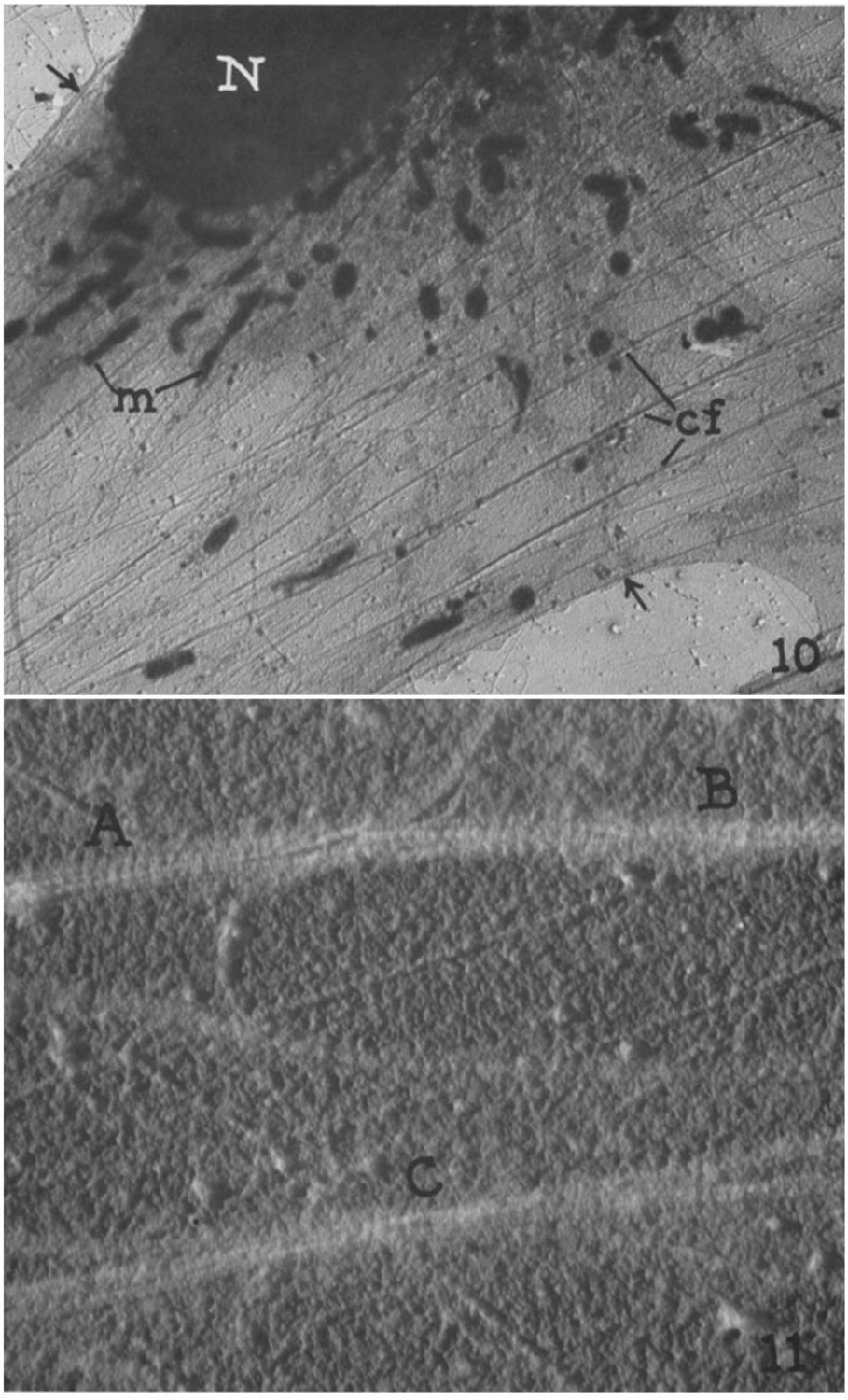


(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 71

FIG. 10. Electron micrograph of a portion of a fibroblast from a 72-hour culture of 9-day chick embryo skin; cell margins indicated by arrows. Cell components, such as the nucleus (*N*) and mitochondria (*m*), are easily identified. Fiber bundles (*cf*) are more or less evenly spaced and in nearly parallel array over the cell surface. Preparation fixed for 10 minutes with vapors of 2 per cent OsO_4 and chromium shadowed. × 5000.

FIG. 11. Portion of the surface of a fibroblast taken from a 72-hour culture. Two fiber bundles are evident. The bundle at the top of the picture is more advanced in its development. At *A* the unit fibrils show a well developed periodicity, which, however, is less evident in the same bundle at *B*. The unit fibrils in the other bundles at *C* are less well shown. × 27,000.



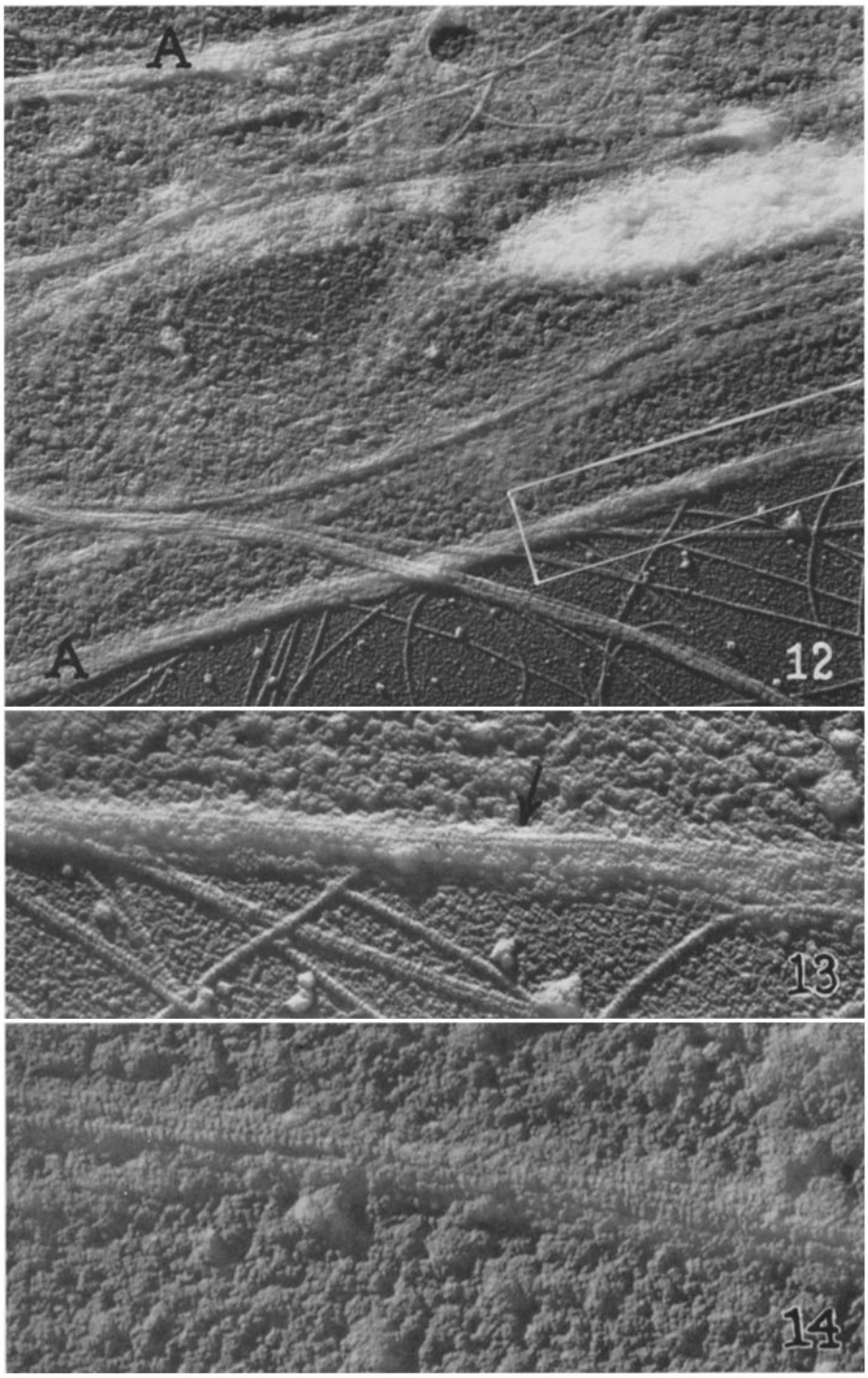
(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 72

FIG. 12. Portion of the tapered tip of a fibroblast from a 72-hour-old culture. The two margins (*A*) of this cell are dense, a condition deriving apparently from a thickening of the cell cortex. × 17,000.

FIG. 13. A greater enlargement of the rectangular area outlined in Fig. 12, showing the fine fibrils (at arrow) developing as part of the marginal thickenings of the fibroblast. × 36,000.

FIG. 14. Micrograph of a small portion of a fibroblast surface taken from a 72-hour-old culture. The intimate relation between the developing fiber bundle and the cell surface is to be noted. × 56,000.

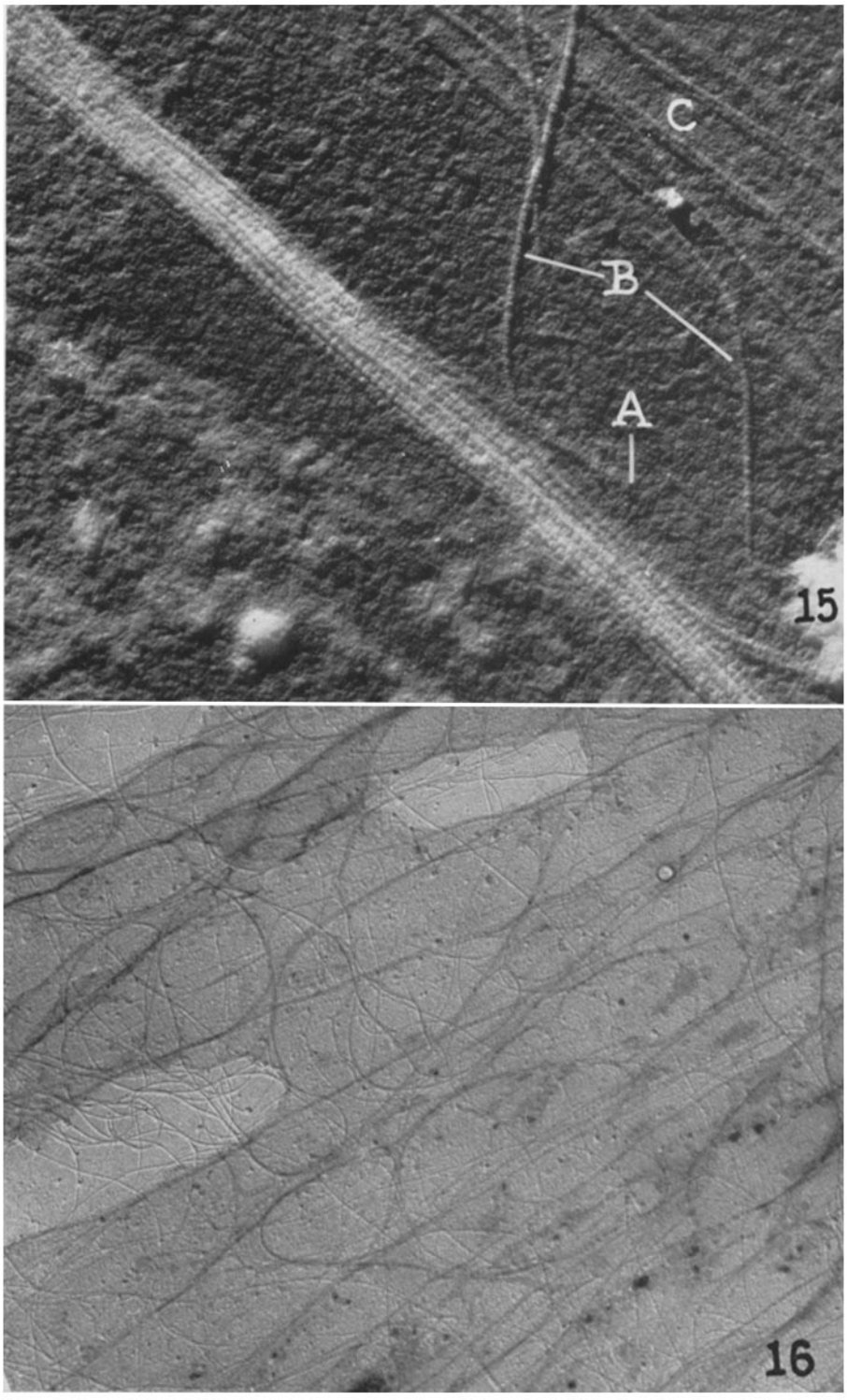


(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 73

FIG. 15. Area of the surface of a fibroblast from a 72-hour culture. A prominent, well developed fiber bundle, made up of striated unit fibrils of collagen, runs diagonally across the image. Some fibers of the bundle appears to run into the cell surface at *A*. Isolated or paired unit fibrils can be seen associated with the cell surface at *B* and *C*. × 23,500.

FIG. 16. Micrograph of part of a fibroblast taken from a 96-hour culture. Many slender fiber bundles and unit fibrils follow circular courses which coincide with ridges of surface material. × 6000.



(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 74

FIG. 17. Image of an area of a fibroblast surface. Material accumulating as ridges on the cell surface is evident at *A*. In some instances these ridges show no evidence of fibrils; in other cases the ridge is continuous with a well defined fiber. × 10,000.

FIG. 18. Small area of fibroblast surface including a straight bundle of extremely slender (early) fibrils (*B*) and an arc-shaped ridge of surface material (*A*). The latter is an integral part of the cell surface (arrow) and hows one or two fine fibrils at one point in its contour. × 30,000.

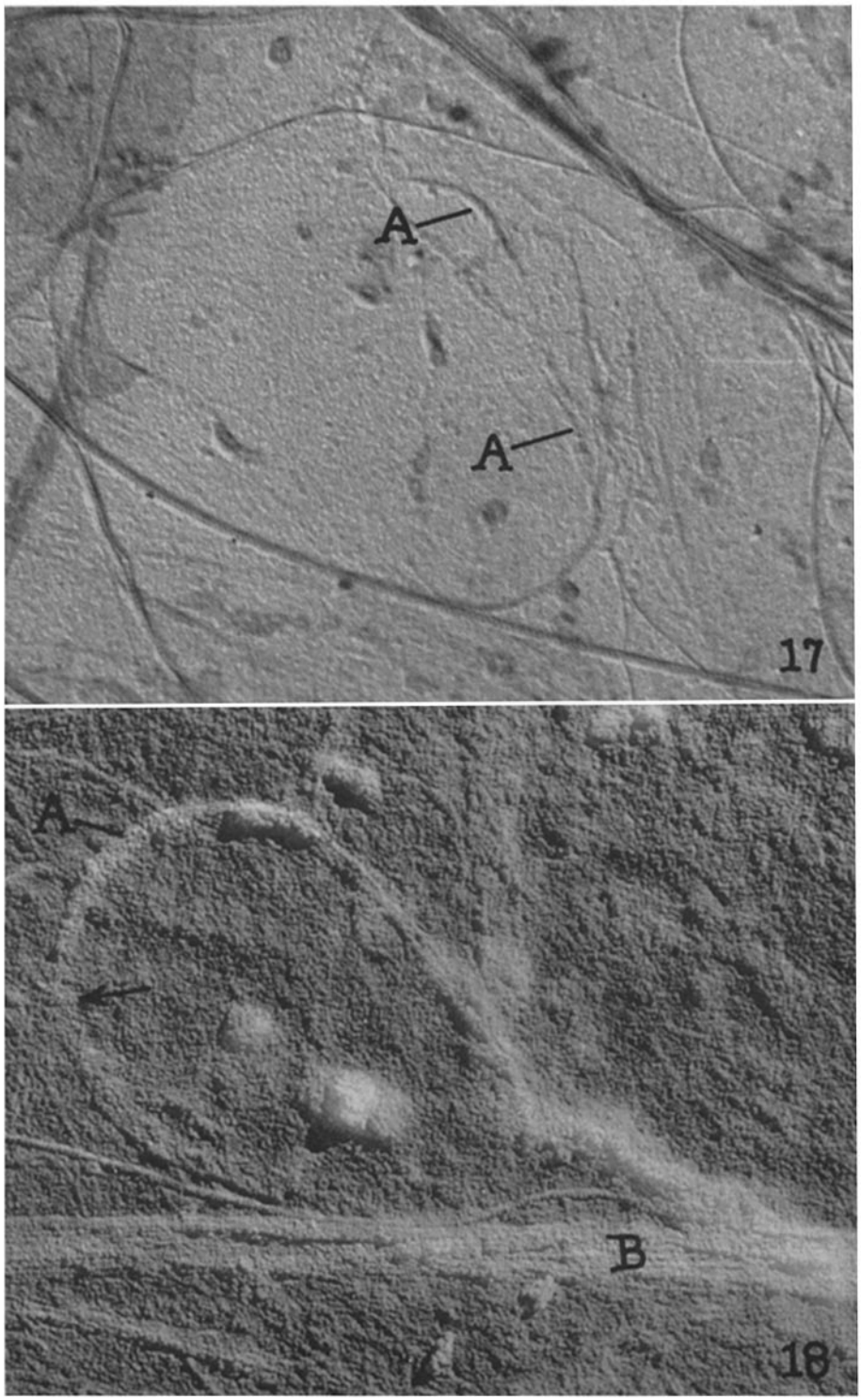


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PLATE 75

FIG. 19. Electron micrograph of a portion of a fibroblast from a 6-day-old culture. The cell margin is indicated by arrows. Almost the entire surface of the cell appears to be contributing to fiber formation. × 23,000.

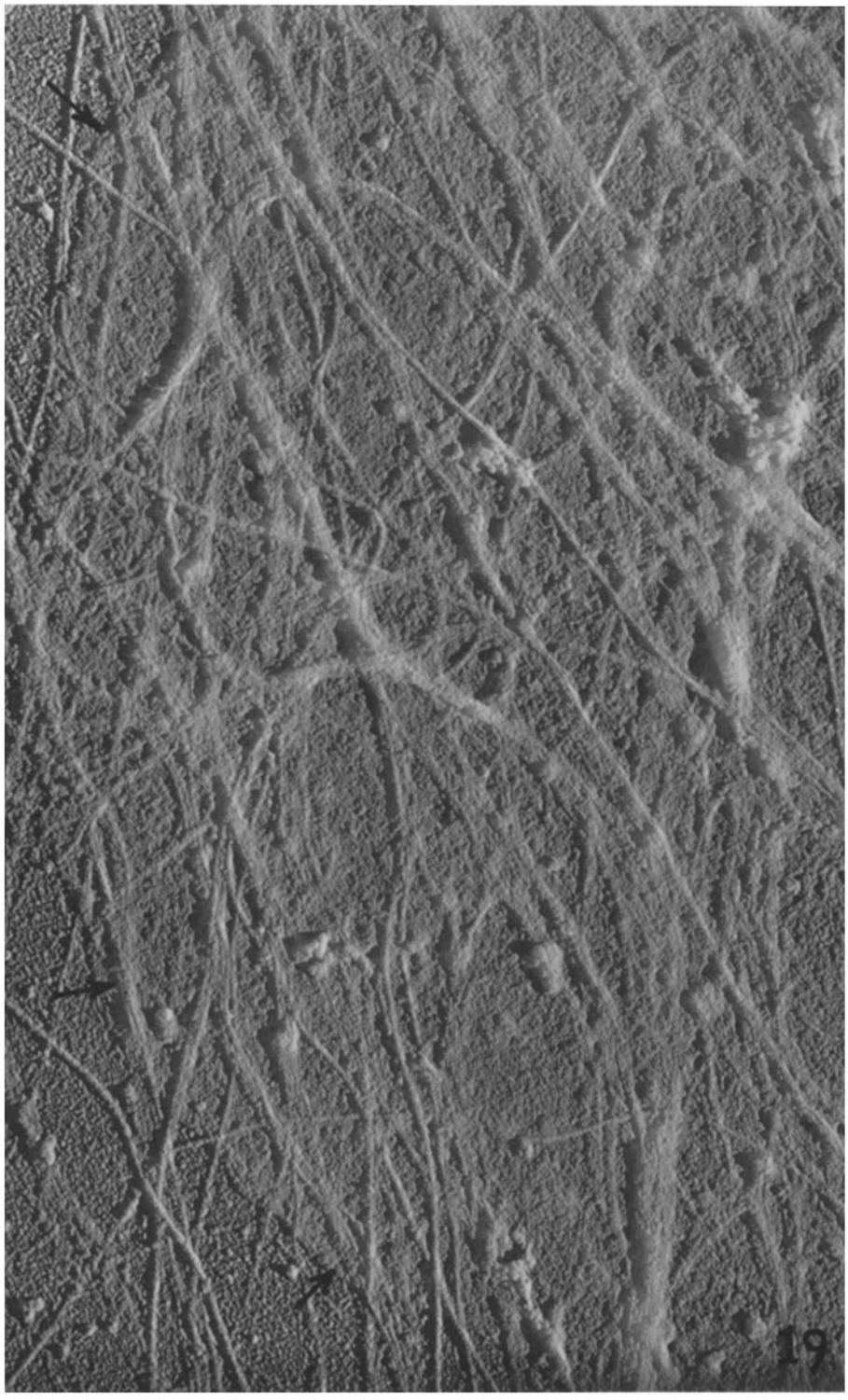

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PLATE 76

FIG. 20. Micrograph of a fibroblast surface from a 72-hour-old culture. Sheet-like condensations of fiber-forming material are evident at *A*. It is likely that this material represents the earliest morphological evidence of collagen fibers at the cell surface. × 34,000.

FIG. 21. Photomicrograph of a section of 14-day-old chick embryo skin. The tissue was fixed in neutral formalin, silver impregnated (Gomori's method), and counterstained with hematoxylin and eosin. Fibrous arrays in the dermis (*C*) and the "basement membrane" (*B*) appear heavily impregnated with silver. × 600.

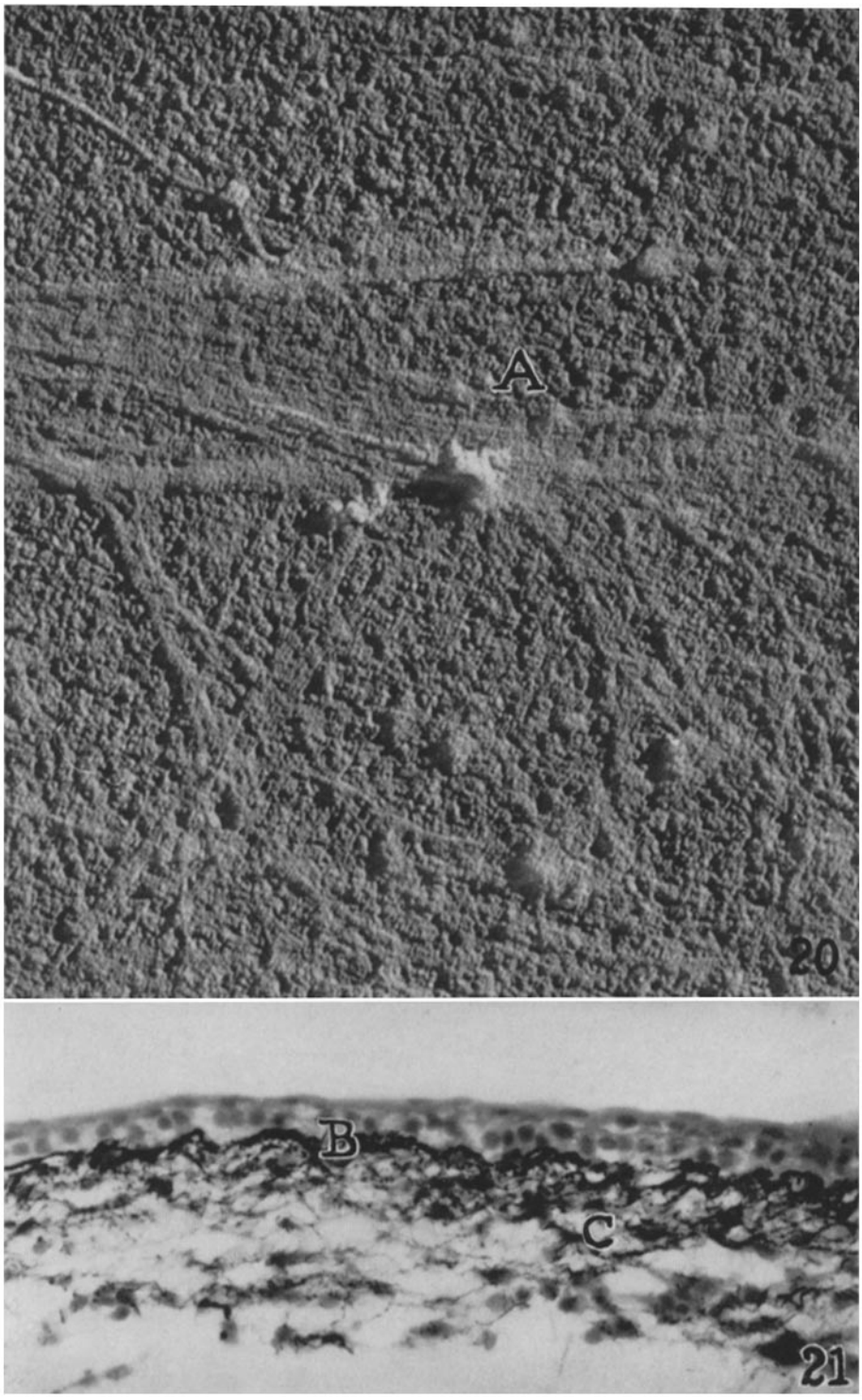

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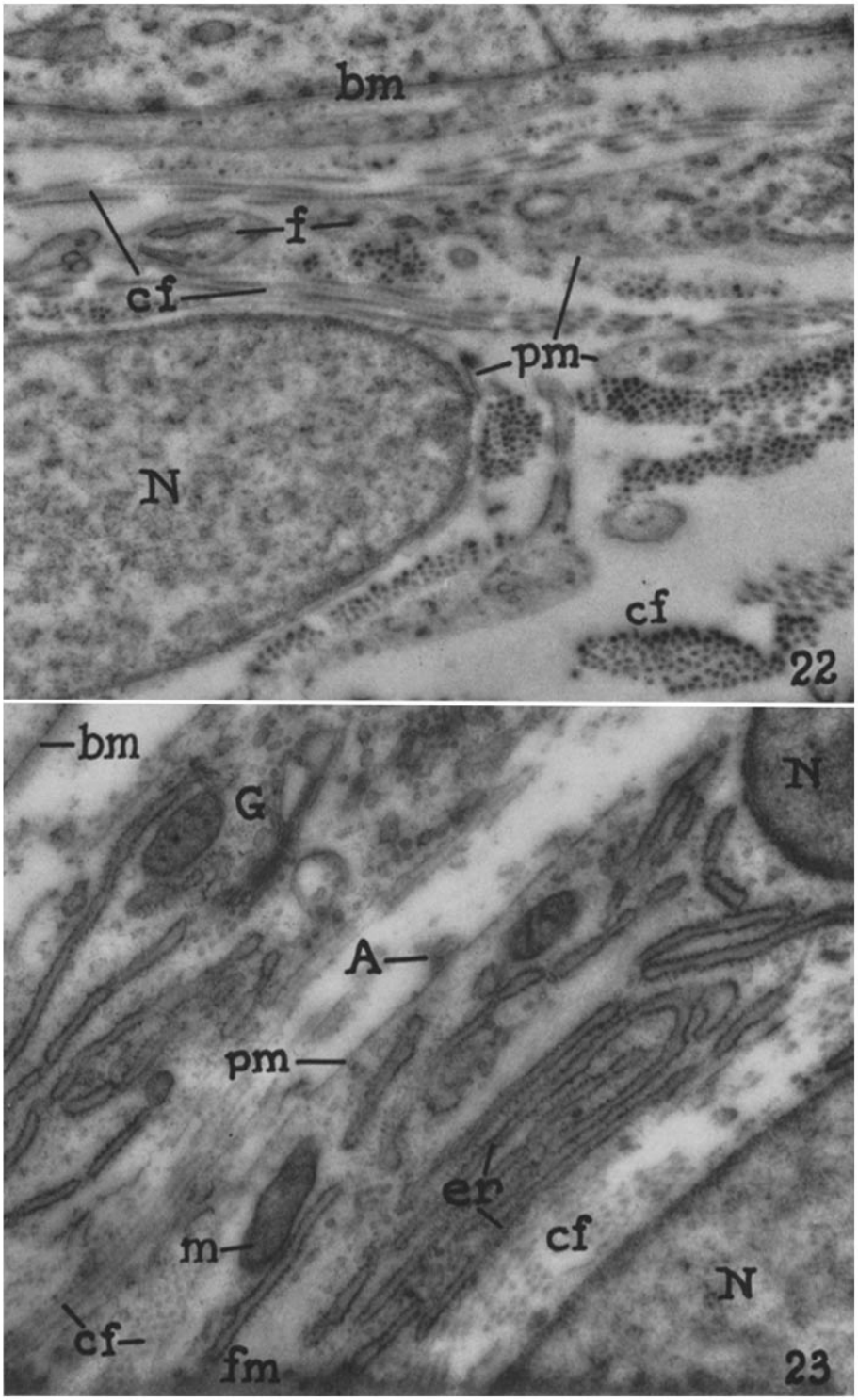
PLATE 77

FIG. 22. Electron micrograph of a thin section of 16-day-old chick embryo skin. The basement membrane (*bm*) is evident at the top of the picture. Below this, in the dermis, sections of pseudopodia (*f*), nucleus (*N*), and plasma membrane (*pm*) of fibroblasts are evident. Extracellular collagen fibers (*cf*) are shown in both cross- and longitudinal images. × 16,000.

FIG. 23. Electron micrograph of a thin section of fibroblasts of the dermis of 14-day-old chick embryo skin. The basement membrane (*bm*) is shown in the upper left hand corner. The cytoplasm of the fibroblasts contains sections of mitochondria (*m*), Golgi component (*G*), as well as the very prominent elements of the endoplasmic reticulum (*er*). Abundant collagen fibers (*cf*) are found dispersed in the intercellular spaces. In the cytoplasm of the fibroblast which runs from the lower left to the upper right of the picture, a fibrous matrix (*fm*) is present. The small mass of greater density (*A*) on the outer surface of the plasma membrane (*pm*) of this fibroblast is closely associated with a small bundle of extracellular collagen fibrils. × 33,000.



(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 78

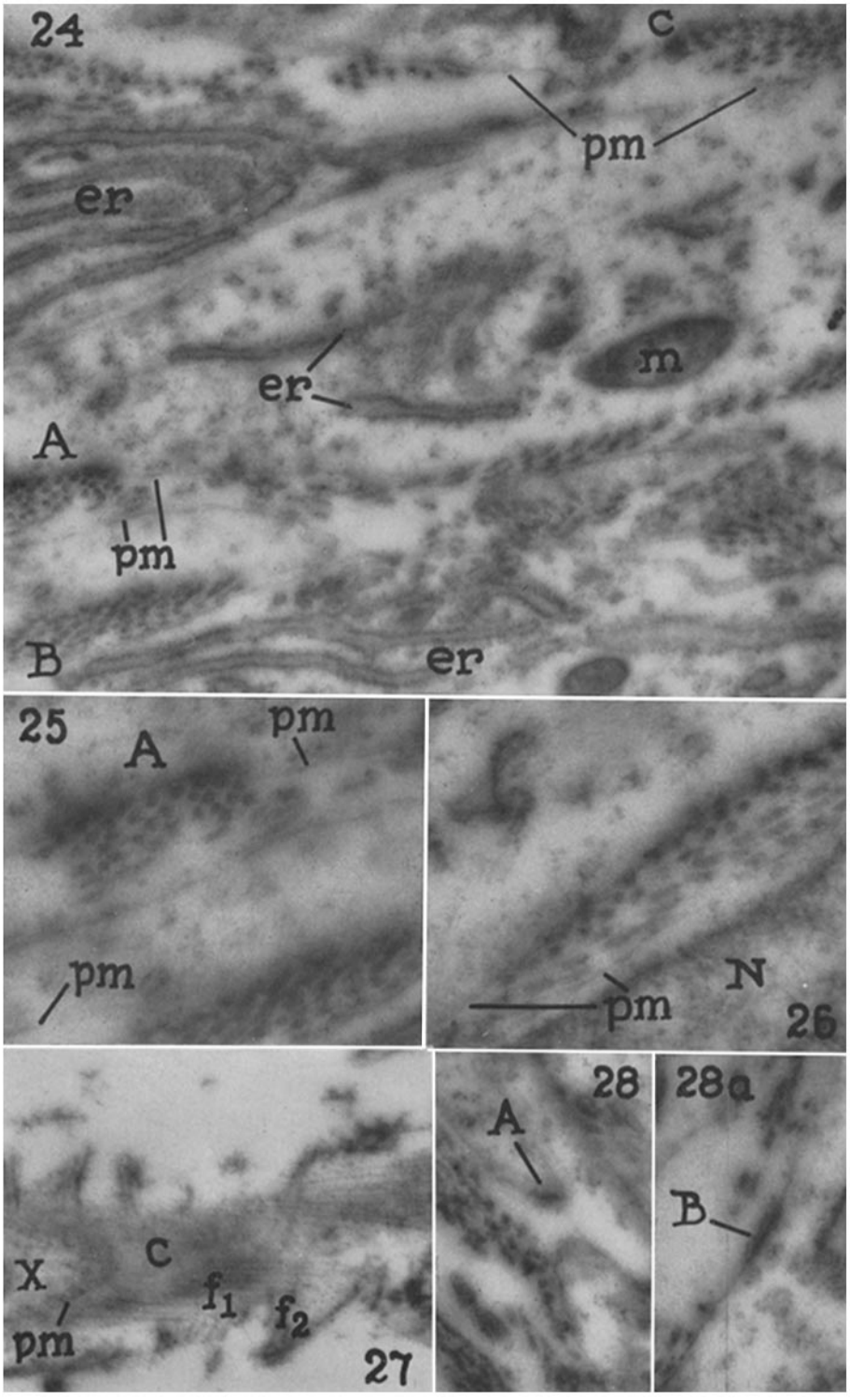
FIG. 24. Electron micrograph from a thin section of the dermis of a 14-day-old chick embryo. Sections through portions of several fibroblasts are evident in the field and may be identified by the cytoplasmic elements shown (mitochondria, *m*, endoplasmic reticulum, *er*). The regions *A*, *B*, and *C* represent places where a localized band of cortical density within the cell coincides with an extracellular fiber bundle. The plasma membrane (*pm*) may or may not be evident at these points. × 20,000.

FIG. 25. Detail of area *A* of Fig. 24. The plasma membrane (*pm*) is difficult to identify at the sites where the fiber bundles and the cell surface are contiguous. × 47,000.

FIG. 26. Shows a small portion of two cells, with collagen fibers in the intercellular space. A fragment of the nucleus (*N*) of one cell plus its plasma membrane (*pm*) are readily identified. The plasma membrane of the other fibroblast can be seen only at the left of the picture. The condensation of material along the surface of this cell is interpreted as contributing to fiber formation. × 51,000.

FIG. 27. A section cut tangential to the cell (*X*), causing the plasma membrane (*pm*) to appear as a wide, poorly defined band. Adjacent to the membrane there is unorganized material (*c*), from which parallel arrays of collagen fibrils (f_1) seem to be developing. Other thicker fibers (f_2) are observed to run at right angles to the first fibrils (f_1) lying in the unorganized matrix (*c*). × 19,000.

FIGS. 28 and 28 *a*. Two portions of the same cell process from a section of 14-day-old chick embryo dermis. Two dense regions (*A* and *B*) show the following components: an extracellular condensation of amorphous material, a thickening of the plasma membrane, and an intracellular cortical condensation. These we regard as representing cross-sections through the earliest cell-surface thickenings leading to fiber production. × 20,000.



(Porter and Pappas: Collagen formation by fibroblasts)

PLATE 79

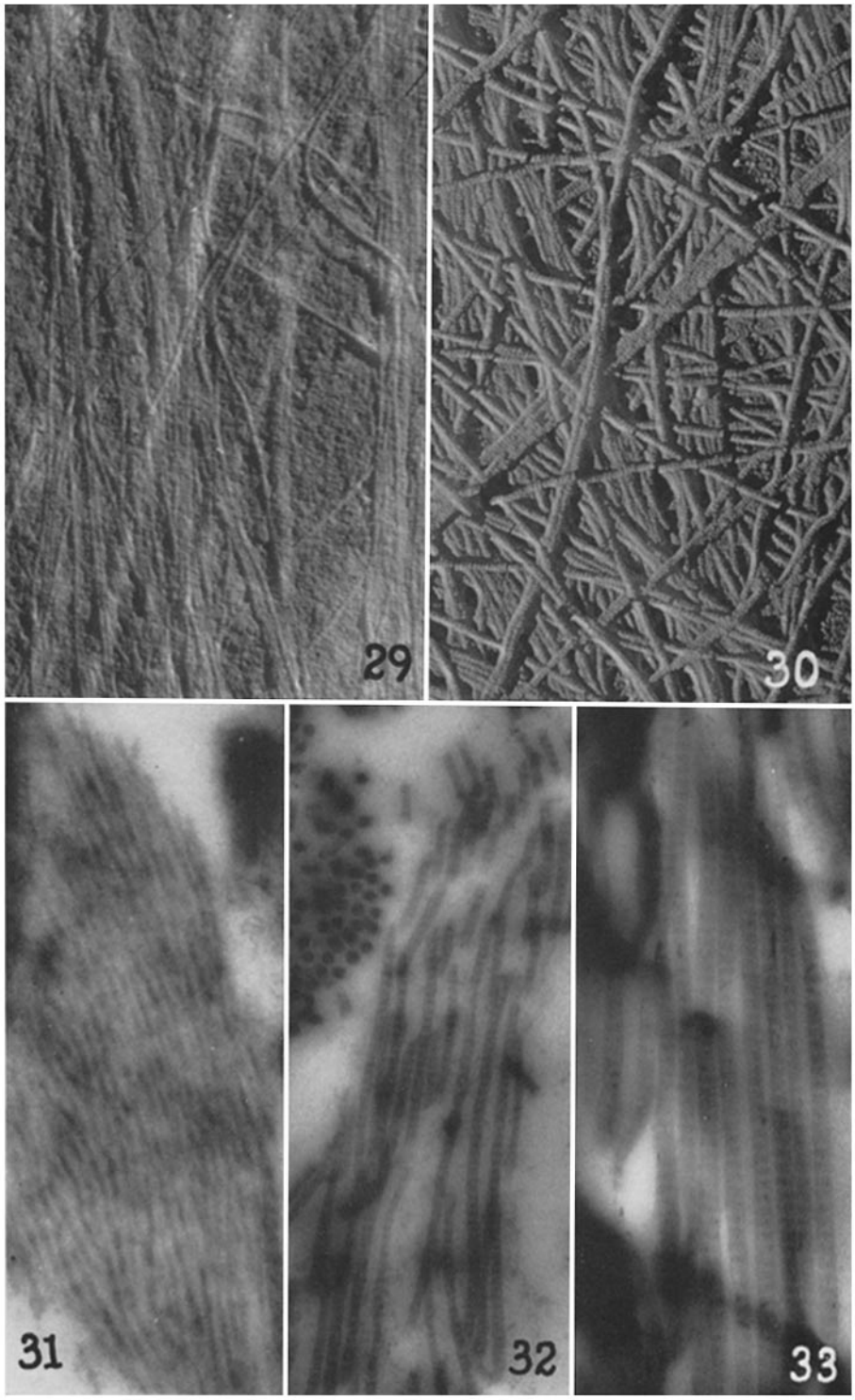
FIG. 29. Micrograph of a portion of a fibroblast surface from a 3-day-old culture of chick embryo skin, grown in Locke-bouillon medium. The diameters of the collagen fibrils average approximately 280 A. × 25,000.

FIG. 30. Micrograph of collagen fibers (and unit fibrils) from a 6-day-old culture grown in Locke-bouillon medium. The width of these fibers averages approximately 520 A. × 25,000.

FIG. 31. Electron micrograph of a thin section through a fiber bundle of the skin of a 12-day-old chick embryo. The diameter of the fibrils measures approximately 250 A. × 40,000.

FIG. 32. Collagen (unit) fibrils from 18-day-old chick embryo skin. The fibril width averages approximately 400 A. × 40,000.

FIG. 33. Collagen (unit) fibrils from adult chick skin. Fibril diameter here varies from 700 to 800 A. × 40,000.



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