WOUND HEALING AND COLLAGEN FORMATION

I. Sequential Changes in Components of Guinea

Pig Skin Wounds Observed in the Electron Microscope

RUSSELL ROSS, D.D.S., and EARL P. BENDITT, M.D

From the Department of Pathology, University of Washington, Seattle

ABSTRACT

The regular sequence encountered in healing guinea pig skin wounds has been examined by methods of light and electron microscopy. Observations on cell populations, their fine structure, and fibril formation in the connective tissue have been made. Linear incisions in the skin of normal female guinea pigs weighing 300 to 350 grams were allowed to heal. The wounds were then excised, fixed with buffered 2 per cent osmium tetroxide, and postfixed in neutral buffered formalin, at 16 and 24 hours and at 3, 5, 9, and 14 days after wounding. They were then embedded in epoxy resin. In the inflammatory phase the exudate observed in the early wounds consists largely of polymorphonuclear neutrophilic leukocytes, macrophages, fibrin, and free extracellular organelles from the disrupted inflammatory cells. These organelles later appear in vacuoles in the cytoplasm of the macrophages. Fibroblasts first appear at 24 hours, and show extensive development and dilatation of the endoplasmic reticulum, which sometimes contains moderately dense flocculent material. In addition, these fibroblasts have enlarged mitochondria and condensations of filamentous material within the cytoplasm near the cell surface. Occasional myelin figures and moderately dense, 0.5 to 1.0 micron bodies are found within the cytoplasm of the early fibroblasts. Collagen fibrils are first seen at 3 days extracellularly near the cell surfaces. They appear at the later times in two populations of sizes. With increasing wound age the fibroblasts retain their morphology and the wounds decrease in cellularity concomitantly with the formation of increasing amounts of collagen. Several proposed mechanisms of collagen fibril formation are discussed in relation to the observed phenomena. The problem of correlating fibril diameter with the appearance of the periodic structure of collagen in relation to the minimal size fibril which would be anticipated to display this appearance is discussed.

INTRODUCTION

Wound healing is a common and fundamental pathologic process. The connective tissue component of wound healing is its most prominent portion. Despite numerous investigations over many years, there are still large gaps in our understanding of the mechanism of collagen fiber formation by fibroblasts *in vivo* during the wound healing process.

The early description of the connective tissue cell by Schwann (1) led to differing views regarding the relationship of this cell to fibrogenesis. Although many investigators assumed this cell type to be responsible for fibrogenesis, it was not until 1940 that Stearns (2, 3) clearly demonstrated, through the use of the transparent ear chamber, that the fibroblast is indispensable for

collagen formation in granulation tissue. Schwann (1), Remak (4), and Schultze (5) believed that the connective tissue fibers developed directly from the cell by splitting away from the cytoplasm; whereas Virchow (6) and Kölliker (7) thought that the cells themselves secreted the fibrils or their precursors. These precursors were then thought to crystallize or gel within the intercellular space. In her investigations Stearns (2, 3) described cytoplasmic granules which she believed contained the precursor for the fibrous collagen which was found extracellularly. Other workers (8–11) have used cell-free extracts of collagen and reconstituted collagen fibrils from these solutions *in vitro*. The fibrils which formed resembled native collagen when examined both by x-ray diffraction and by electron microscopy. In their investigations of collagen formation in tissue culture, Porter and Vanamee (12), Porter (13), and Porter and Pappas (14) found the earliest fibrils at the cell surface. They postulated an enzyme or template mechanism for fibril polymerization from soluble precursors in this region. Other investigators (15–21) have described variations from the earlier findings including primary intracytoplasmic fibrils, which they presumed to be some form of collagen. Findings such as collagen fibrils within membrane-bounded spaces as noted in some of these papers could also be interpreted as either cell invaginations or intracytoplasmic fibrils. Gieseking (22, 23) has studied the fine structure of collagen formation in chick embryos and points to the above problems of interpretation. Technical problems of tissue preservation in some of these earlier electron microscopic studies have added confusion due to artifacts which arose in the process of tissue preparation.

Certain requirements for collagen formation in the intact animal may be different from those in the embryo or in tissue culture. It has been found by Woessner and Gould (24) that chick embryo fibroblasts grown in culture do not require ascorbic acid for collagen synthesis. Gould *et al.* (25) also found that guinea pig embryonic tooth buds continued to form dentin when implanted in the anterior chamber of the eye of totally scorbutic guinea pigs. Most recent electron microscopic studies of collagen formation have been concerned with chick embryos or tissue culture (12–19, 22, 23). The healing wound in guinea pigs has been chosen as the experimental model in this study, primarily because we are interested in *in vivo*

collagen formation. Such wounds are easily manipulated, and with them the various stages of the process can be studied sequentially in the same animal. The *in vivo* model avoids the very abnormal though simplified situation presented by fibrogenesis in tissue culture.

MATERIALS AND METHODS

Tissue Preparation for Light Microscopy

Wound healing was observed in female guinea pigs weighing 300 to 350 gm which were fed a standard guinea pig diet (Nutritional Biochemicals) containing 20 mg ascorbic acid per 20 gm of diet. The hair was removed from the backs of the animals with an electric clipper. The wounds were produced with a round rotary 5 mm punch biopsy tool and extended to the muscle. After 1, 3, 5, 9, and 13 days, the wounds were removed. They were halved, fixed in neutral buffered formalin at room temperature for 36 hours, and embedded in paraffin. Several staining procedures were used, including hematoxylin and eosin, the van Gieson stain, Mallory's connective tissue stain, Wilder's reticulin stain, the periodic acid–Schiff technique and aqueous toluidine blue.

Cells were counted using an ocular grid. Grading of zero to three was based on numbers of cells per high power field, a grade of one being equivalent to 2 to 4 cells, grade two representing 4 to 10 cells, and grade three representing more than 10 cells.

Tissue Preparation for Electron Microscopy

Eight incisions, each approximately 1 cm long, were made in the skin with a scalpel, extending to the muscle, and were left to heal by primary intention. The wounds were then removed by taking an ellipse of skin around each wound at the following time intervals after wounding: 16 and 24 hours, and 3, 5, 9, and 14 days. After the ellipse was removed, the wounds were cut transversely with razor blades and transverse sections approximately 1 mm thick by 2 mm. in length were fixed in 2 per cent osmium tetroxide buffered with s-collidine (48) (pH 7.4) at 0°C for 1 hour. The tissues were then fixed again in neutral buffered formalin at 0°C for 1 hour. Following this they were dehydrated in a graded series of ethanol (50 to 100 per cent). The tissue was placed in two changes of propylene oxide for 10 minutes each, and then in a 1:1 mixture of propylene oxide and epoxy resin anhydride mixture (Epon 812) for 3 hours. The blocks were infiltrated overnight in the epoxy resin with added accelerator (DMP-30). They were then embedded in the resin in capsules, and the resin was polymerized at 60°C overnight (26).
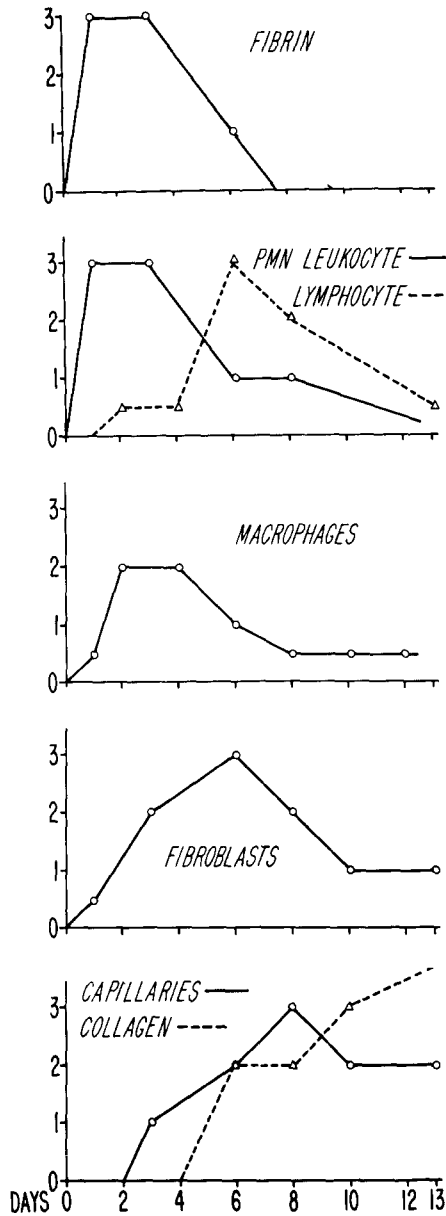
FIGURE 1

This graph contains the elements of the healing wound in the normal guinea pig as determined from 0 to 13 days. The various cellular and acellular elements are graded on a 0 to 3 basis using numbers of cells per high power field as a basis for grading. These observations were made upon wounds which were prepared for routine light microscopy and stained with hematoxylin and eosin, van Gieson connective tissue stain, phosphotungstic acid–hematoxylin, and Wilder's reticulin stain.

The orientation of the tissue specimens could be easily seen in the blocks. If the specimens were not positioned so that transverse sections could be made, they were remounted with epoxy resin on aluminum blocks, and were then trimmed so that only the wound and immediately adjacent connective tissue were left. Sections were cut with glass knives on a Porter-Blum microtome. They were mounted on carbon-coated grids and stained for 4 hours in 1.5 per cent uranyl acetate. For each section an immediately adjacent 1 to 2 micron section was taken and mounted on a glass slide. The various cells examined in the electron microscope could be located in the adjacent thick section by phase microscopy or in toluidine blue–stained sections (27), permitting determination of cellular orientation within the wound. Electron microscopy was done with a modified RCA EMU-2C.

OBSERVATIONS[1]

The sequence of events observed in the *light microscope* in the healing wounds is as follows (Fig. 1): Within 12 hours the wound is filled with an exudate consisting of polymorphonuclear leukocytes, erythrocytes, macrophages, and fibrin. The numbers of these cells increase for the first 24 hours to a maximum and then rapidly decrease after the 3rd day. A few mononuclear cells, called by various observers histiocytes, monocytes, or "polyblasts" (28), are present at 24 hours. Some observers (31, 32) have even called these cells fibroblasts; however, the morphologic distinction of a fibroblast at this level of resolution is possible only by the association of the cell with active collagen formation. This cannot be ascertained at this time in the healing sequence. The numbers of fibroblasts rapidly increase to the 6th or 7th day. By the 5th day active collagen formation is heralded by the appearance of argyrophilic fibers,

[1] We have used the term "macrophage" in this paper for a cell type with the ultrastructural characteristics described in a later section. The term "histiocyte" has been used interchangeably with "macrophage" for this cell type by several investigators (28, 65). The monocytes of hematologic origin probably play a role as macrophages in the wound exudate and possibly as progenitors for fibroblasts. Since it is impossible to differentiate between macrophages of tissue origin and macrophages from the blood, the term "macrophage" is here applied to *all* cells which can be clearly differentiated from the fibroblasts. These macrophages resemble one another in certain characteristics and frequently, though not alawys, show evidence of phagocytic activity.

and by the 7th day by van Gieson–positive fibers which course parallel to the surface of the fibroblasts. At approximately 9 days the number of fibroblasts begins to decrease and the collagen concomitantly increases in amount and becomes coarser in appearance.

As observed in the *electron microscope*, the various cell types seen at the various time intervals are the same as those seen in the light microscope preparations and corroborate the findings of earlier workers (31, 32). The sequence of events is as follows.

AT 16 HOURS: In comparison with the light microscope findings, the 16 hour specimens display a network of fibrin which is seen extensively throughout the entire wound. The characteristic banding of the fibrin can occasionally be seen. Lying adjacent to the strands of fibrin are numerous polymorphonuclear neutrophilic leukocytes, and a few erythrocytes and macrophages (Fig. 2). An additional component which is present throughout the area is spherical bodies ranging in size from 1000 A to 3000 A (Fig. 3). Many of these clearly resemble the organelles of the intact polymorphonuclear leukocytes, some of which appear to have an electron opaque center with a less dense periphery while others are very dense throughout. Each of these bodies is bounded by a single membrane. In some regions, groups of these structures are arranged around a multilobed nucleus and have no plasma membrane surrounding them. This apparently represents a stage of fragmentation of the leukocytes. The macrophages which are present contain many of the same membrane-bounded bodies within cytoplasmic vacuoles (Fig. 4). These we interpret as lysosomes as described by de Duve (37) and Novikoff (49).

AT 24 HOURS: By 24 hours the cell population has changed. The number of polymorphonuclear leukocytes is still large and many are seen in the process of disruption. The macrophages are more numerous, and contain not only the spherical bodies mentioned above, but also dark, irregularly shaped, homogeneous bodies which are not apparently membrane-bounded, and well developed myelin figures (Fig. 5). Several cells which can be recognized as fibroblasts are seen at this time (Fig. 6). These cells are characterized by an extensive, large, well developed, dilated endoplasmic reticulum. The mitochondria exhibit variations in size and matrix density from cell to cell. The cristae are short and irregular, and vary in size. An occasional myelin figure can be seen within the cytoplasm of the fibroblasts. Numerous small rosettes of granules, presumed to be ribosomes, are scattered throughout the cells, as well as fine filaments which are approximately 35 to 50 A wide and of indeterminate length. Fibrin is still present extracellularly.

AT 3 DAYS: At 3 days fibrin is still present and many polymorphonuclear leukocytes are seen. The cell population consists largely of macrophages and fibroblasts. The numbers of fibroblasts are increased relatively to the other cells present. Small numbers of collagen fibrils are now found extracellularly. Many of the fibroblasts contain large numbers of intracytoplasmic filamentous structures, ranging from 50 to 70 A in width. The mitochondria appear as before but a few individual mitochondria seem to have lost one of their two limiting membranes in a particular region (Fig. 7). An occasional amorphous, moderately dense body with an irregular outline can also be seen within the cytoplasm of these cells. These bodies are not clearly surrounded by limiting membranes. In some fibroblasts the endoplasmic reticulum appears to be more extensively developed than in others.

AT 5 DAYS: On the 5th day large numbers of extracellular collagen fibrils with their characteristic 600 to 700 A periodicity can be seen (Fig. 8). The majority of the population of these fibrils

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FIGURE 2

Micrograph of a section of a 16 hour wound. Two polymorphonuclear neutrophilic leukocytes (*P*) and a basophilic leukocyte (*BA*) are present together with strands of fibrin (*fb*) and numerous bodies (*o*) similar in size and appearance to the cytoplasmic organelles (*o*) of the polymorphonuclear leukocytes. Some of the granules (*g*) of the basophil appear to be composed of membranes stacked in parallel array *ca.* 150 A apart. Nuclei at *N.* Membrane breaks apparent in several regions are the result of sectioning damage. × 21,000.
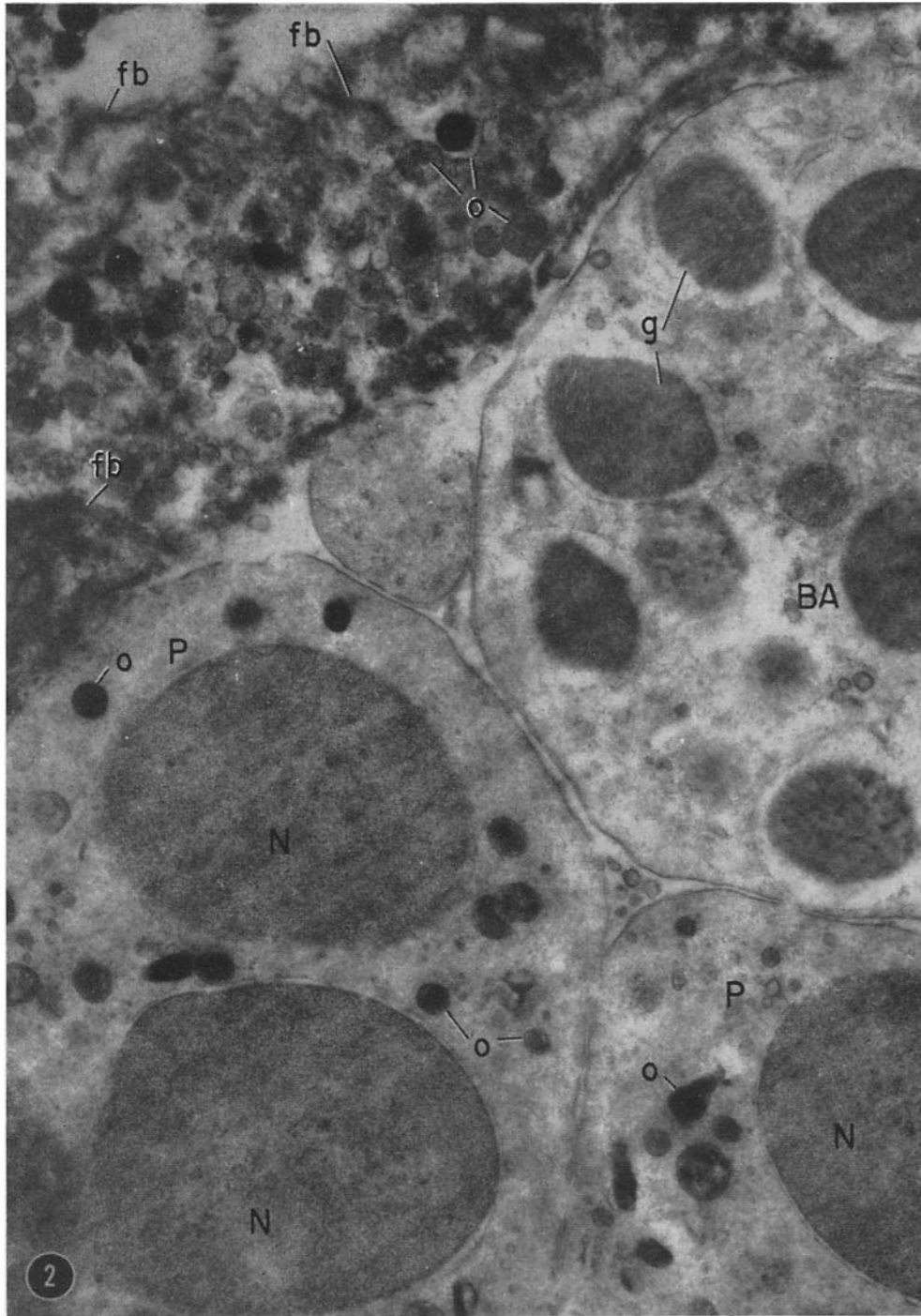
are 400 A in width. The fibrils immediately adjacent to the cells are narrower (200 A) than those which are farther displaced from them. The system of the endoplasmic reticulum appears to be abundant and very well differentiated in many of the fibroblasts (Fig. 8), and the profiles of the cisternae form a continuous series of chan-

nels. Because of the dilatation in many regions, the area occupied by the cisternae appears to approximate that occupied by the remaining cytoplasm. Intracytoplasmic 50 to 70 A filaments can be seen in the cytoplasm between the cisternae of the endoplasmic reticulum. No banded fibrils are present within the cytoplasm of the fibroblasts.
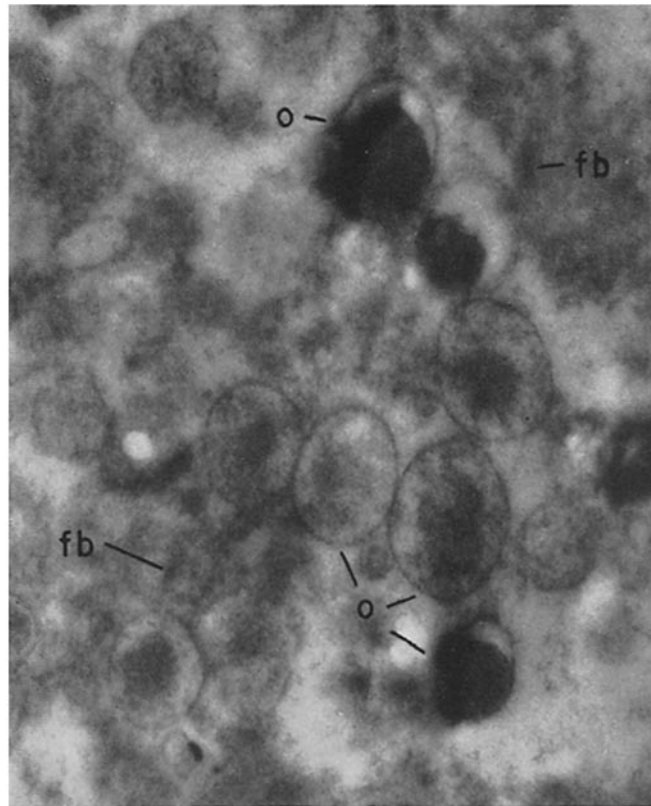

FIGURE 3

Micrograph of a section of a 16 hour wound in an area in which the membrane-bounded granules (o) and vesicular structures of the disrupted polymorphonuclear leukocytes, and fibrin (fb) are present. The unit membrane structure is evident in several of these bodies. × 71,000.

FIGURE 4

Micrograph of a section of a 16 hour wound in an area where three macrophages are present. Numerous vacuoles (v), possibly lysosomes, containing the ingested organelles of the disrupted polymorphonuclear leukocytes as well as myelin figures are evident. Irregularly shaped, very electron opaque bodies (b), not obviously membrane-bounded, are present. Mitochondria (m), Golgi complex (G) and a portion of the nucleus (N) of one of these cells can be seen. Extracellular fibrin (fb) is present in several areas. The endoplasmic reticulum (er) is scanty with small cisternae. × 18,000
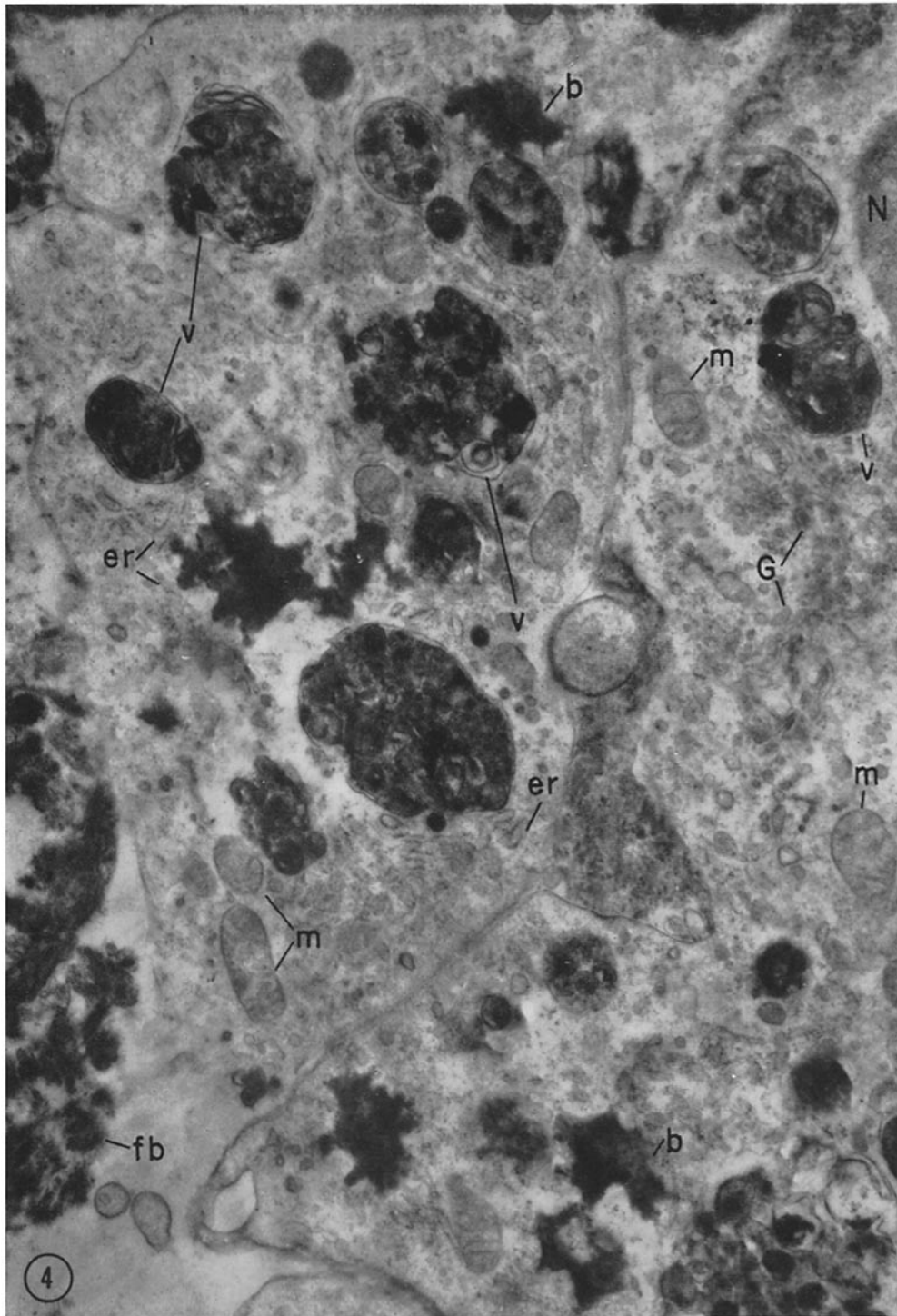
At 9 Days: The component cells and their relative numbers are the same at 9 days (Fig. 9) as at 5 days and the collagen fibrils continue to increase in width. At this time essentially two populations of sizes are present. They are in the range of ca. 250 A and ca. 1000 A in width. The cytoplasm of the fibroblasts contains dense aggregates of material near the cell surface. At higher magnification (Fig. 10) these aggregates are seen to be formed by accumulations of fine parallel filaments. The filaments range from 20 A to 80 A in width and do not exhibit a banded structure.

At 14 Days: The morphologic features at 14 days parallel those of the earlier time periods. The collagen fibrils are wider and occur in two populations of sizes (Figs. 11 and 12). Both populations contain fibrils of uniform width, one averaging ca. 500 A, and the other ca. 1300 A (Fig. 13). The endoplasmic reticulum continues to be extensively developed and the cisternae of many of the cells are still widely dilated. The peripheral aggregates of filamentous material continue to appear within the cytoplasm of the fibroblasts.

DISCUSSION

Many of the changes taking place in the sequence of events which comprise wound healing have been dealt with by Johnson and McMinn (29), Jackson (30), Edwards and Dunphy (31), and Arey (32). The earliest of these changes, the inflammatory reaction, has been studied by surprisingly few investigators in its specific relation to the events which follow. And the role of this inflammatory reaction in subsequent connective tissue formation has been little stressed (33–36). It has been claimed that upon depression of this reaction, collagen formation is delayed in onset and decreased in amount. In view of this it is noted that our experimental findings indicate that in the first 16 to 24 hours many of the polymorphonuclear

leukocytes become fragmented and their cytoplasmic granules are ingested by the macrophages in the area. Cohn and Hirsch (51) have demonstrated that the granules of the polymorphonuclear leukocyte of the rabbit, and presumably of other species, contain many enzymes, particularly acid phosphatase, alkaline phosphatase, nucleotidase, ribonuclease, and deoxyribonuclease. They believed that the properties of these granules are analogous to those of the liver lysosomes. What role, if any, these bodies and other constituents play as stimulants to protein synthesis or as chemotactic agents is unknown. It is interesting that these bodies seem to be taken up by macrophages and to appear in vacuoles, presumably lysosomes (37, 49). Some of the ingested material seen in the macrophages may also be the breakdown products of erythrocytes; however, no evidence of actual erythrophagocytosis was seen in this material (50).

Fibroblast Differentiation

The problem of determining which cell or cells act as precursors to the fibroblast has plagued many investigators (28, 38, 39). A study such as this may give some evidence to substantiate some of the claims relating to either the "polyblast," lymphocyte, or blood-borne monocyte as being the progenitor of the fibroblast. Relevant to this is the observation that the only cells which have any development of their endoplasmic reticulum early in the phase of the healing process are the macrophages. These macrophages are characterized predominantly by (a) the presence of numerous vacuoles containing ingested matter; (b) the presence of myelin figures in some regions within these vacuoles; (c) irregularly shaped, very dense amorphous masses of varying size; and (d) cisternal profiles of the endoplasmic reticulum which are generally narrow and short at 16 hours. The ob-

Figure 5

Micrograph of a section of a 24 hour wound. Parts of two macrophages (MAC) are present in which vacuoles containing myelin figures (v) and irregularly shaped electron opaque bodies (b) are seen. Mitochondria (m), Golgi complex (G), nuclei (N), and endoplasmic reticulum (er) are evident within these cells. Parts of a fibroblast (F) and a polymorphonuclear neutrophilic leukocyte (P) are observed, as well as organelles (o) from disrupted polymorponuclear leukocytes, lying between these cells. Remnant collagen (c) fibrils from the dermis are present in one area. × 17,000.
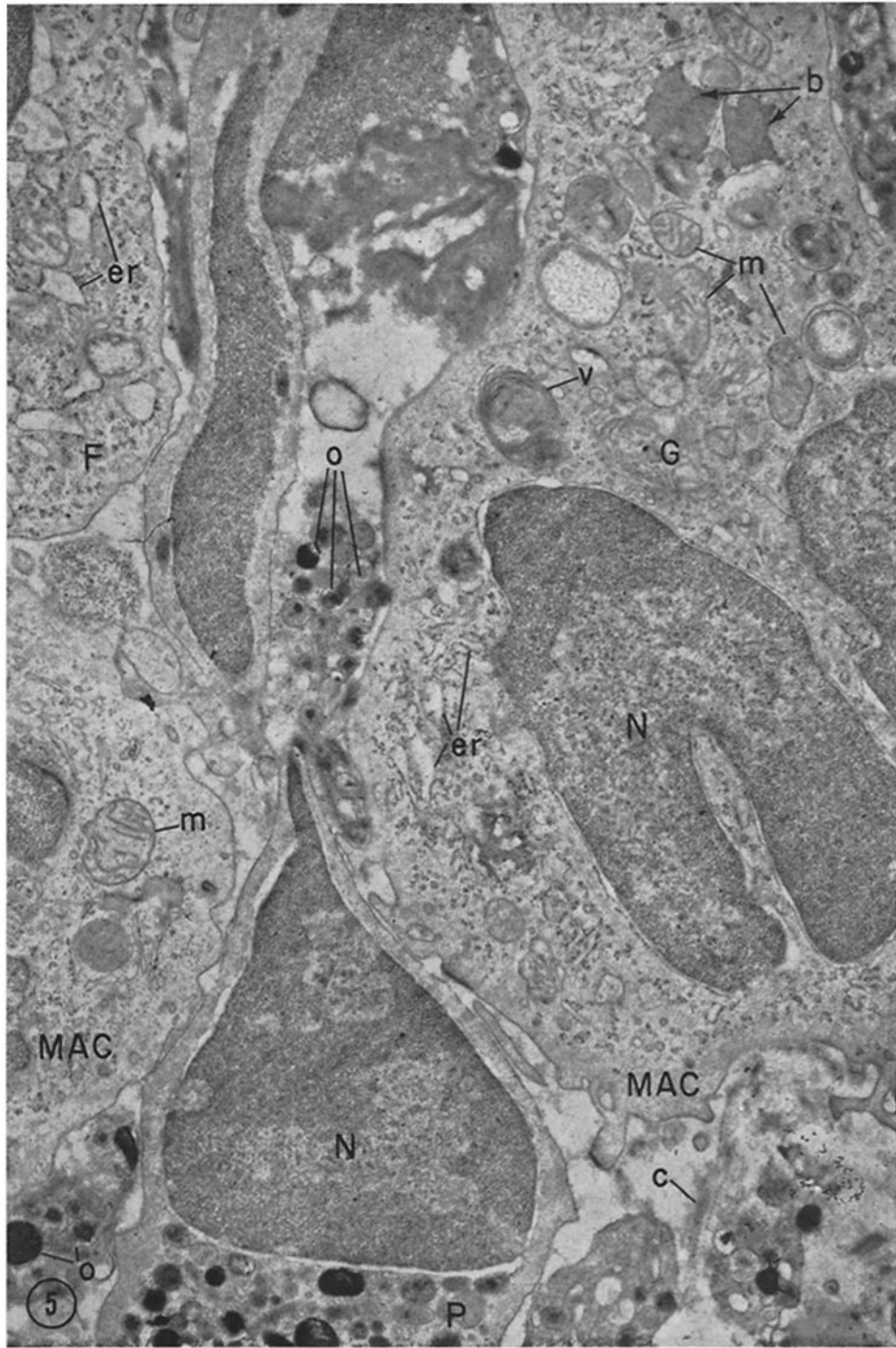
servation that by 24 hours the endoplasmic reticulum of these macrophages appears to be more extensively developed suggests a transition from these cells to fibroblasts, which are increasing in numbers after 24 hours. Consistent with this interpretation is the presence in recognizable fibroblasts of occasional intracytoplasmic bodies similar to those characteristic of the macrophages.

The fibroblasts are differentiated from the macrophages by (a) extensive development and dilatation of the endoplasmic reticulum; (b) enlarged mitochondria with irregular, small cristae and a pale intercristal space; (c) intracytoplasmic 50 to 70 A filaments; (d) marginal intracytoplasmic filamentous condensations consisting of groups of approximately 20 to 80 A filaments; (e) occasional irregularly shaped, amorphous dense bodies; and (f) rare myelin figures. By far the most striking feature in these cells is the extensive and dilated endoplasmic reticulum. In many cells, particularly from the 5th day on, the profiles of the cisternae form a large anastomosing network of channels. The ribosomes lining the exterior of these spaces are missing in some areas. In those areas where the wall of a cisterna is tangentially cut, these particles can be seen to be numerous and closely spaced. In some cells the cisternae contain a moderately dense flocculent material, whereas in others they do not. Many of these characteristics have been reported by several workers (22, 23, 59).

The work of Palade and others (Palade, 40; Palade and Siekevitz, 41; Campbell, 42) indicates that the rough surfaced endoplasmic reticulum is intimately associated with protein synthesis. The means of release of this protein, or proteins, was not apparent morphologically in the present study. No obvious communications of the cisternae with the exterior are visible. Numerous small cytoplasmic vesicles and caveolae, possibly indicative of pinocytotic activity, can be seen. Whether these are related to the endoplasmic reticulum or to some other activity of the cells is unknown.

Fibrillogenesis

An important question is that of the actual site and sequence of collagen fibril formation. The hypotheses concerning the possible mechanism of collagen fibril formation, namely, (a) intracytoplasmic fibril formation with the loss of a part of the cytoplasm of the cell to the milieu (15, 16, 21, 54, 58); (b) secretion of a soluble precursor which then "gels" or polymerizes extracellularly independently of the cell (9, 16, 63); and (c) extracellular fibril polymerization, at the cell surface, dependent upon the cell (12–14), can now be discussed in the light of the present findings.

INTRACYTOPLASMIC FORMATION: As noted earlier, many investigators have made claims for intracytoplasmic collagen fibril formation. We are unable to confirm these findings. One of the probable reasons for the previous findings is the relatively poor tissue preservation sometimes inherent in methacrylate embedding, used in many of these experiments. Yardley *et al.* (15) comment on the difficulty of defining the plasma membrane of the fibroblasts in their study on fibrillogenesis. Chapman (58) also refers to breaks in the plasma membrane of fibroblasts in the carrageenin granuloma. The granulomata in his

FIGURE 6

Micrograph of a section of a 24 hour wound. One of the first cells at this time to be clearly characterized as a fibroblast (F) is present in the center. A tangential section of the nucleus (N), with demonstration of continuity of nuclear envelope with endoplasmic reticulum (arrow), a myelin figure (v), and a single irregular electron opaque body (b) are present within the cytoplasm of this cell. The mitochondria (m) appear to be enlarged and the cisternal profiles of the endoplasmic reticulum (er) are numerous and dilated. Numerous rosettes of RNP particles are present throughout the cell cytoplasm. Fine filaments (f) are present within the cytoplasm in one region near the border of the cell, where similar appearing extracellular filaments are also evident. The irregularity of the surface of this cell with its many processes and indentations is a common finding. Parts of several polymorphonuclear neutrophilic leukocytes (P) surround this cell in this region. × 22,000.
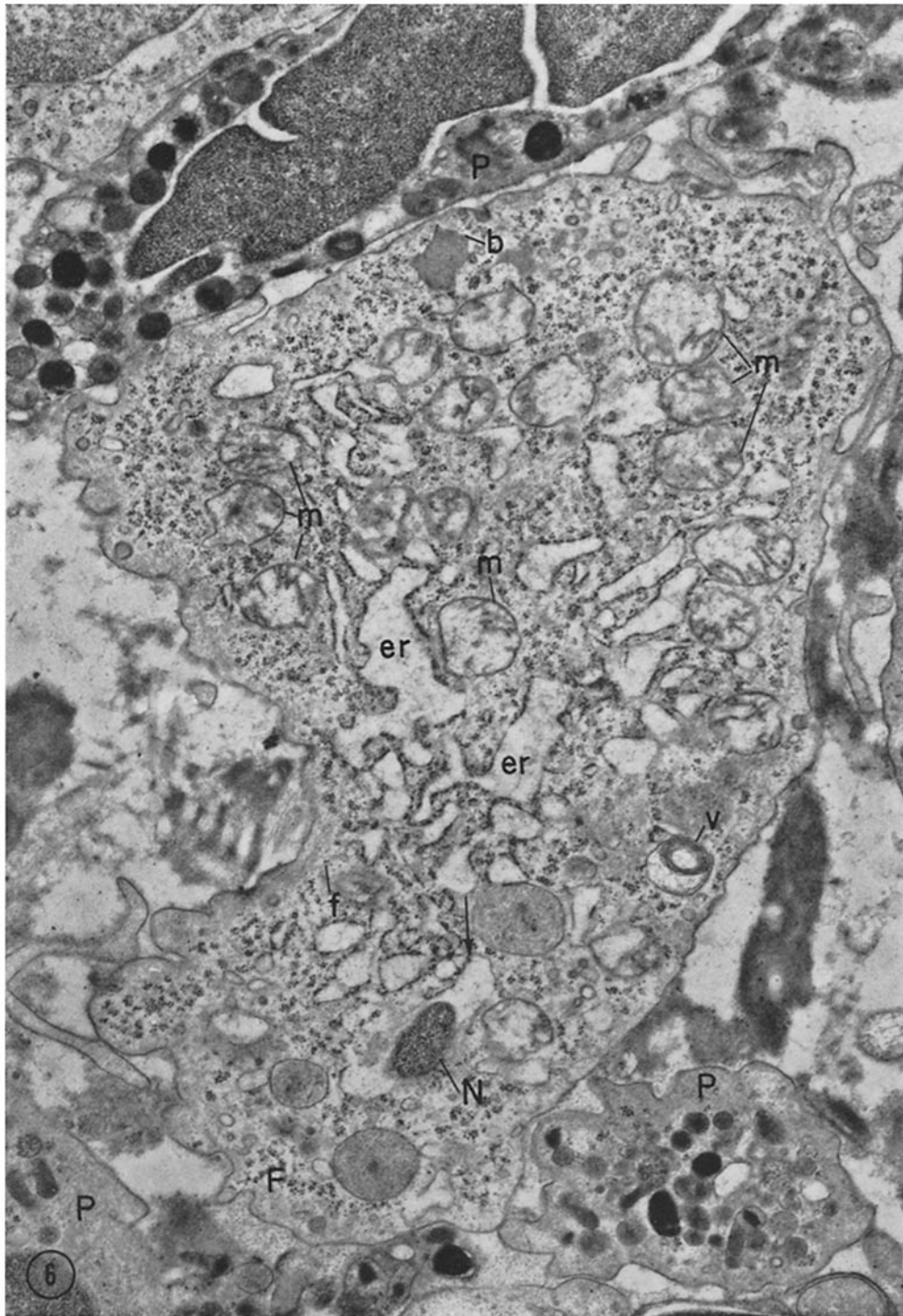
study were embedded in Araldite. In the present investigation the plasma membrane can be clearly seen in all cells. The only area in which this membrane is not well defined, but is clearly intact, is related to regions in which the cell has been tangentially sectioned. In many areas extracellular collagen fibrils can be seen situated extremely close to the plasma membrane of a cell. When these areas are cut tangentially the fibrils appear to stream from the cytoplasm of the cell. Great caution must be used, therefore, in interpreting such areas as sites of intracytoplasmic fibril formation. It is significant that no discontinuities can be seen in the plasma membrane of the fibroblasts (Figs. 5–13).

What relation the numerous intracytoplasmic filaments, approximately 20 A to 80 A wide, grouped in parallel array near the cell periphery may bear to early collagen formation is not clear. The same question arises for the other intracytoplasmic filaments (50 to 70 A) which are so commonly seen within the fibroblasts. There is no way, at present, of being certain that there is a relationship, since no banding or other characteristic of collagen is evident in these filaments. This problem is discussed further below. In addition, many other cell types, such as glomerular epithelial cells (52), capillary endothelial cells, and mesothelial cells (53), have been shown to contain intracytoplasmic filaments having a similar appearance. The unique feature in the fibroblast is the aggregation of these filaments into parallel arrays near the cell surface (Figs. 9 and 12). The means for releasing them to the extracellular milieu could be, as has been postulated, a breakdown of the plasma membrane and its subsequent re-formation between these aggregates and the remaining cytoplasm (15, 58). If such a mechanism for release of these fibrils were in fact existent, one would expect to find some morphologic evidence of the manner in which the

cell reestablishes continuity of its plasma membrane after fibril release. Several devices could be employed by the cell, such as vesicle formation behind the area, with fusion of the vesicles; pinching off of a portion of the cytoplasm at a narrow region, making reestablishment of continuity feasible; or the emergence of these filaments via vesicular structures. We have not found evidence for any of these possibilities in this study. Consequently we are unable, at this point, to explain the role of these filaments in collagen formation, if they are indeed involved in this process.

Several means for extruding precursor other than actual shedding of part of the cytoplasm of the fibroblasts could be invoked; for example, openings of the cisternae to the exterior, reverse pinocytosis, or passage through the intact cell membrane. Morphologic evidence—seen in significant numbers of observations—of the possible means of release of precursor is found in the small vesicles commonly associated with pinocytosis. There is great difficulty in interpreting this finding, since it is impossible to determine the direction of movement of these structures.

Shedding of cytoplasm as in apocrine secretion is a means by which extrusion of collagen precursors could occur. We have observed, immediately following ascorbic acid therapy to scorbutic guinea pigs, a phenomenon consistent with this idea: structures identical with individual cisternae of endoplasmic reticulum appear lying free in the extracellular spaces. These vesicles are intimately associated with numerous collagen fibrils. This was never seen in the control animals. A similar observation was made by Karrer (54) in his study of the developing chick aorta.

EXTRACELLULAR FORMATION INDEPENDENT OF THE CELL: In relation to the second possible mode of collagen formation, namely, secretion of soluble precursor which polymerizes independ-

FIGURE 7

Micrograph of a section of a 3 day wound. A part of a fibroblast in which the nucleus (N), endoplasmic reticulum (er), and mitochondria (m) are present is shown. Two of the mitochondria contain regions (A) in which one of the outer limiting membranes is not apparent. Filaments (f) approximately 50 A in width are present throughout the cytoplasm of this cell as well as numerous rosettes of ribosomes. × 36,000. The insert (upper right) shows an enlargement of some of the many intracytoplasmic filaments present within these cells. × 80,000.
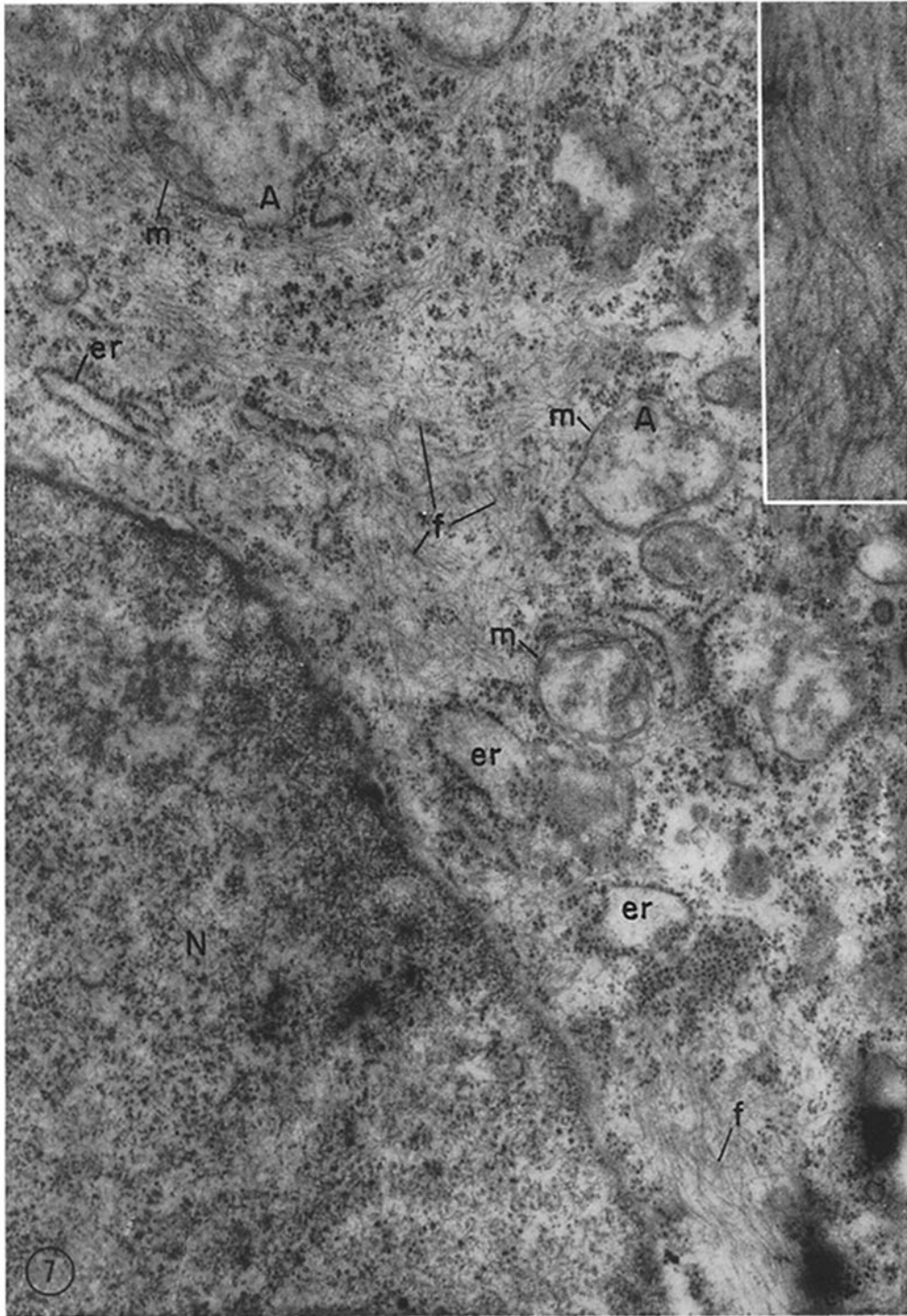
ently of the cell, the work of Gross et al. (9) and Hodge et al. (10) clearly indicates that collagen fibrils can form from a soluble precursor, even in the absence of collagen fibrils. The dimorphic forms of collagen which Hodge and Schmitt (43) found under specific circumstances indicate a possible means for increase in fibril width in an environment in which collagen fibrils are present in combination with soluble precursors. If the cells are secreting such a precursor, this could explain the continuing increase in fibril width with increasing age. The two populations of fibril widths in the later time periods do not appear to be continuous in size, although the diameter of the fibrils in each population is increased at each later time period. At 5 days the majority of the fibrils are in the range of ca. 400 A in width, whereas those nearest the cells are ca. 200 A or less. At 9 days these populations are ca. 250 A and 1000 A, respectively, and at 14 days they are ca. 500 A and 1300 A.

Two possible modes of formation of these fibrils could be consistent with these findings. First, they could be explained by a periodic formation of collagen fibrils in which the wider fibrils were formed at an earlier time, with continuing side-to-side and end-to-end aggregation of precursor molecules. The narrower population of fibrils would, on this basis, have begun to polymerize at a more recent time than the wider ones. Jackson and Bentley (64) have also referred to this possibility. This aggregation and polymerization could be controlled by the cell, either by synthesis and release of precursor at only these intervals, or by continuous synthesis, and release of precursor. With periodic modification of the extracellular milieu, the cell would therefore allow polymerization to take place only during these intervals. A second possible interpretation of this phenomenon might be that the two populations of fibrils form by side-to-side aggregation

of already polymerized smaller fibrils into wider ones. As yet there is no evidence for this type of process.

It is conceivable that a constant remodeling of fibril size is occurring during the course of fibrillogenesis. This also would have an effect upon fibril diameter within any given region. It is not known in what form the collagen precursors might be released to the extracellular environment, if indeed they are secreted in a soluble form. The morphologic study of a phenomenon such as this permits only speculation about these possibilities in the absence of any obvious structural change which can be associated with this activity.

EXTRACELLULAR FORMATION DEPENDENT UPON THE CELL: The third possible mechanism is that of the formation or polymerization of fibrils at the surface of the cell dependent upon the cell itself. This also implies the necessity of the presence of a soluble precursor. Our findings of fine filaments of extracellular collagen near the surface, sometimes related to dense aggregates of material within the cell beneath the plasma membrane, with a regular increase of fibril width with increasing wound age, correlate with those of Porter and Pappas (14).

In summary, therefore, the observations in healing wounds are consistent with secretion of the precursor and polymerization, both free of and dependent upon the fibroblast, as has been hypothesized by Gross et al. (9), Gross (63), Fitton Jackson (16–18), Porter and Vanamee (12), Porter (13), and Porter and Pappas (14). The studies during recovery from scurvy, alluded to previously, point to the possibility of intracytoplasmic formation with shedding of a portion of the cytoplasm as suggested by Yardley et al. (15), Wassermann (21), Karrer (54), and Chapman (58). One or any combination of all three methods may occur in a given situation. Improved techniques in electron microscopy, accompanied by

FIGURE 8

Micrograph of a section of a 5 day wound. A part of a fibroblast demonstrating the extensive dilation and continuity of the cisternae of the endoplasmic reticulum (er) is evident. Part of an enlarged mitochondrion (m) is present. Numerous fibrils of collagen (c) displaying a 600 A periodicity with several interperiod bands are present extracellularly, adjacent to the surface of this cell. The width of the finer fibrils is approximately 190 A. This section was double stained with uranyl acetate and phosphomolybdic acid. × 51,000.
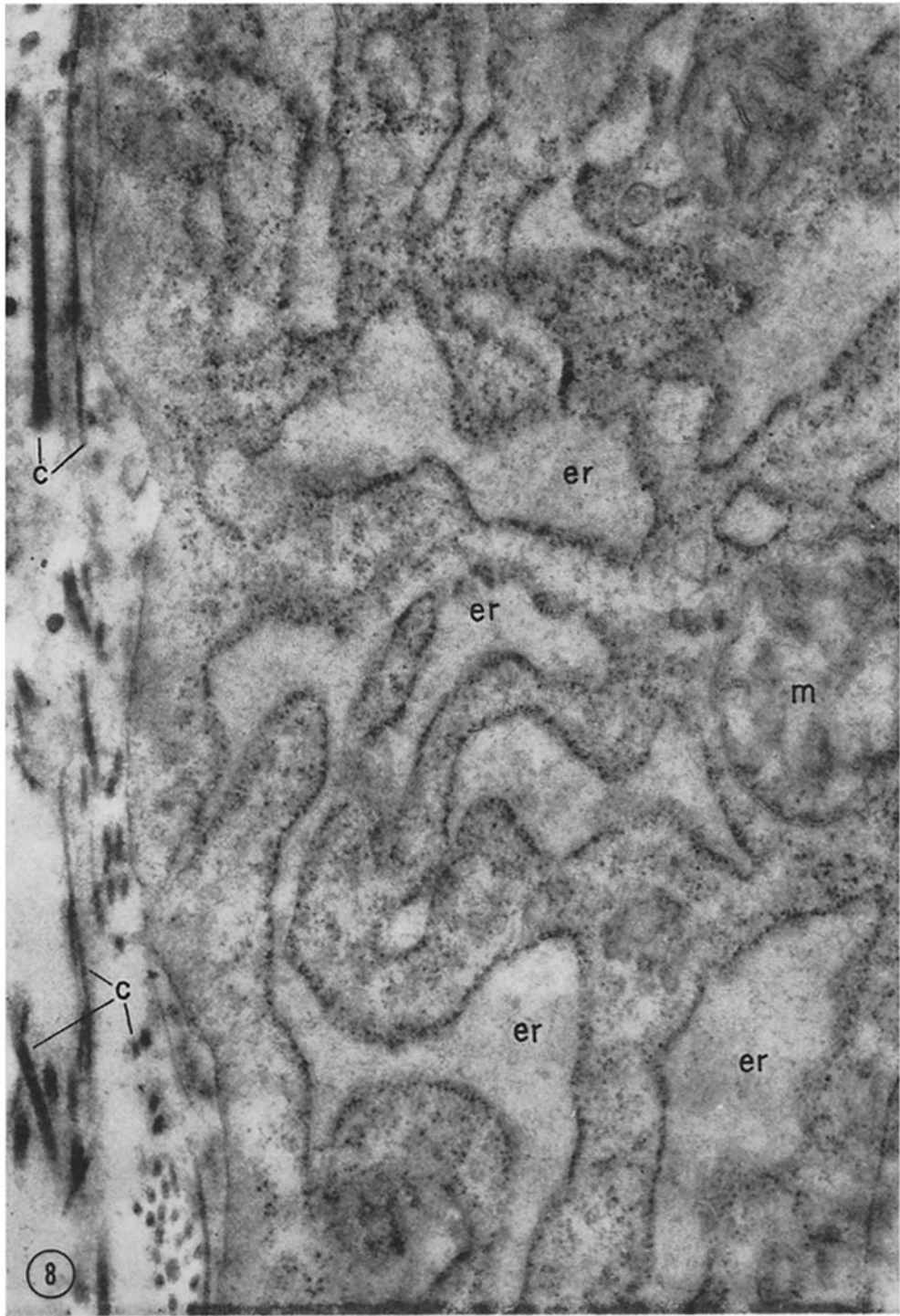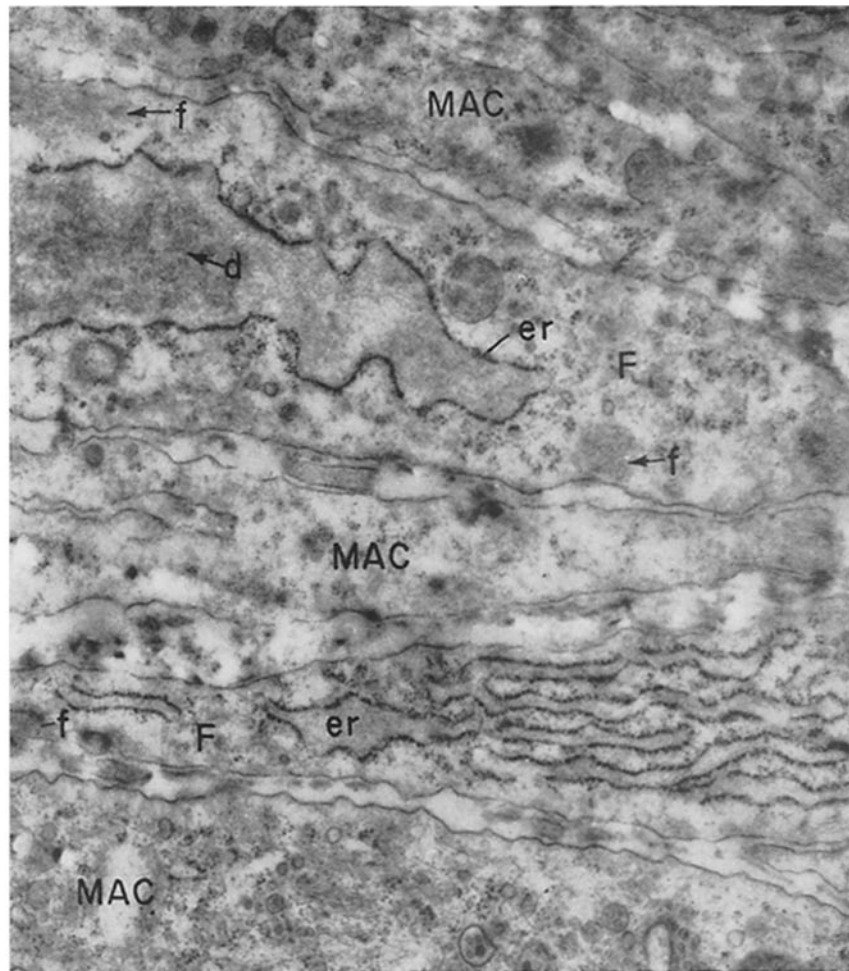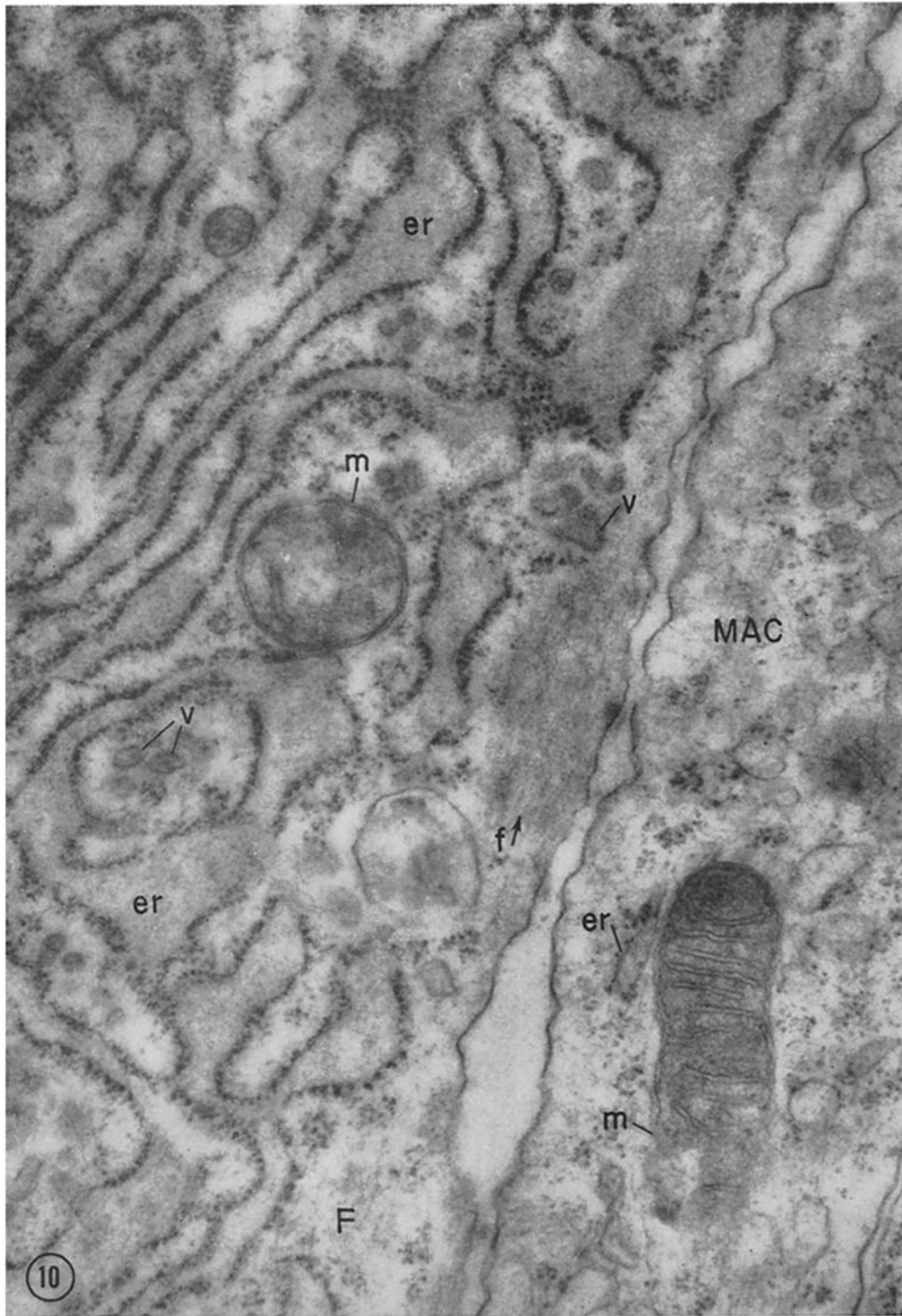
FIGURE 9

Micrograph of a section of a 9 day wound. The two cell types present are clearly seen in this picture. The fibroblasts (F), characterized by the development of their endoplasmic reticulum (er), lie between the macrophages (MAC), which contain numerous vesicles. The greatly dilated cisterna of one of the fibroblasts contains aggregates of moderately dense material (d). Marginal intracytoplasmic filamentous condensations (f) are evident within these fibroblasts. Some of the macrophages contain individual filaments, but marginal condensations of filaments are never seen in these cells. × 21,000.

FIGURE 10

Micrograph of a section of a 9 day wound. Parts of two cells representing the two types seen in Fig. 9 are evident. Mitochondria (m), endoplasmic reticulum (er), and vesicles (v) of the fibroblast (F) are contrasted with those of the macrophage (MAC), which shows a paucity of endoplasmic reticulum. A marginal filamentous condensation (f) typical of the fibroblast is present in one area. The filaments in this condensation range from 20 A to 80 A in width. × 48,000.
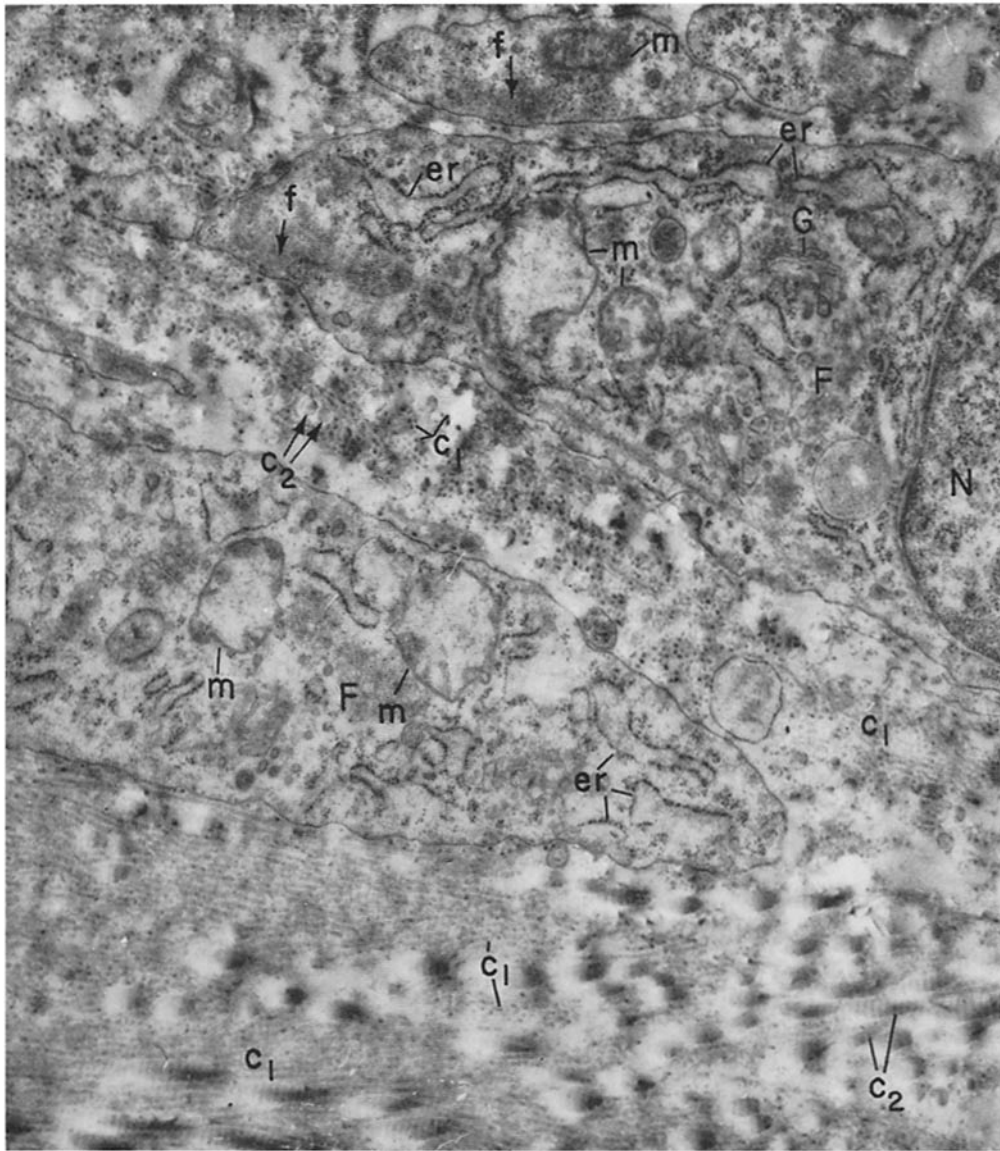
FIGURE 11

Micrograph of a section of a 14 day wound. Parts of several fibroblasts (F) are present. The mitochondria (m) are enlarged; the Golgi complex (G), endoplasmic reticulum (er), nucleus (N), and peripheral filamentous condensations (f) are seen in these cells. Two populations of sizes of collagen fibrils cut transversely (c_1, c_2) are evident between the cells. These fibrils are approximately 250 A and 1000 A in width. $\times$ 23,000.
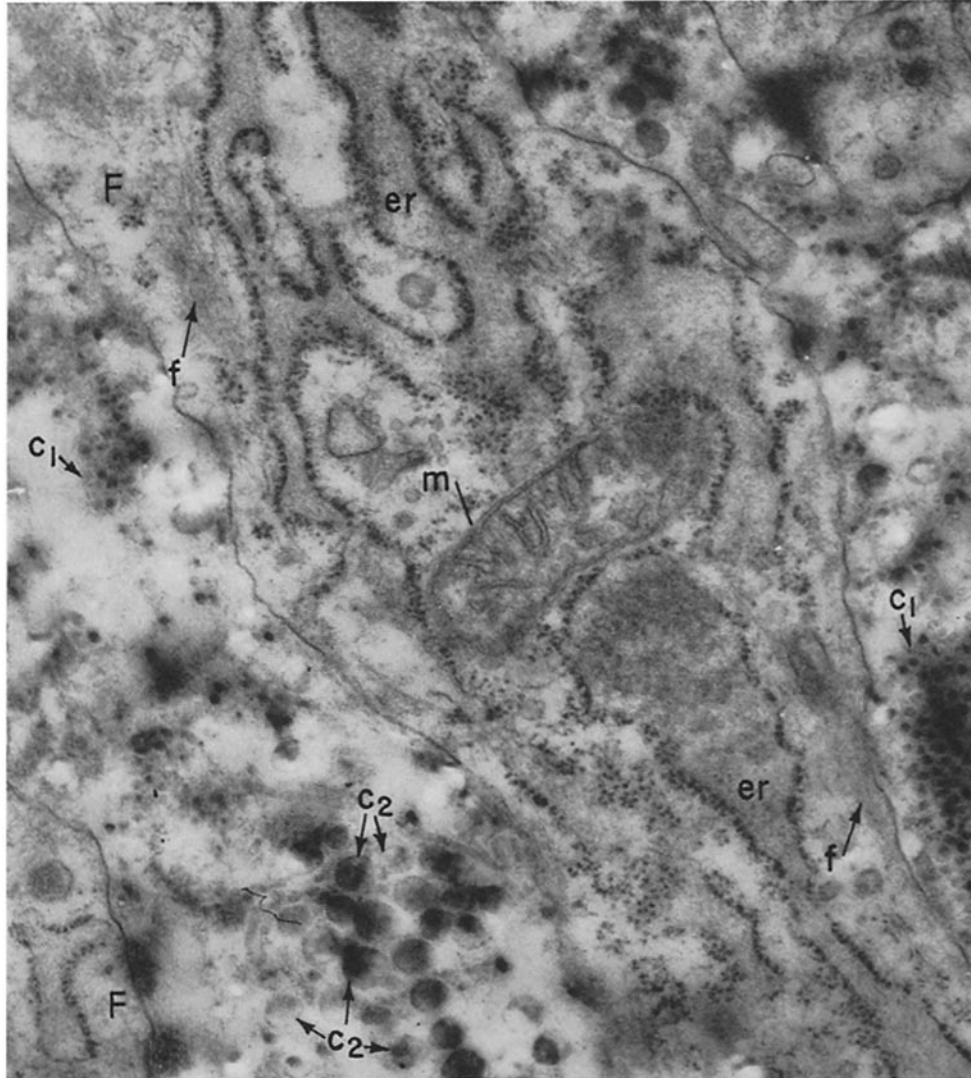
Micrograph of a section of a 14 day wound. A part of a fibroblast (F) containing a mitochondrion (m), dilated endoplasmic reticulum (er), and marginal filamentous condensation (f) appears in the center of the picture. The cisternal contents of the endoplasmic reticulum are denser than the cytoplasmic matrix and appear to consist of short thread-like condensations. The collagen fibrils (c_1, c_2) surrounding these cells are largely cut transversely, although short longitudinal segments are present. The two populations of fibrils are evident in this area as they are in Fig. 11. $\times$ 37,000.

other approaches, should add to our knowledge of this complex phenomenon.

Intracytoplasmic Filaments

The problem of determining whether the intracytoplasmic filaments found by many investiga-

tors in fibroblasts (15, 16, 19–22) are some form of collagen is a difficult one. Several factors must be taken into consideration if one is to identify filaments or small fibrils as collagen by the appearance of a 700 A periodic banding. If the native type of collagen is formed by a side-to-side aggregation in a "quarter-stagger" arrangement

as shown by Hodge and Schmitt (43), it is clear that at least 4 or 5 tropocollagen molecules would be necessary in any given dimension before the "quarter-stagger" arrangement could give the effect of a 700 A periodicity. This assumes the unit molecule of tropocollagen to be 2800 A long and 14 A wide (55, 56). This would necessitate a minimum width of 56 to 70 A before such an arrangement could occur in any given dimension. These figures are based upon the assumption that

the "quarter-stagger" arrangement is one in which *nearest tropocollagen neighbors*, arranged in parallel array, are longitudinally displaced by one-fourth of a tropocollagen length, as explained by Hodge and Schmitt (43). It can be seen in Figs. 11 and 12 that the gross collagen fibril has a cylindrical shape, being approximately round in transverse section. This shape would, of necessity, dictate an arrangement of tropocollagen molecules which could only be found in groups of 4 or 5 in
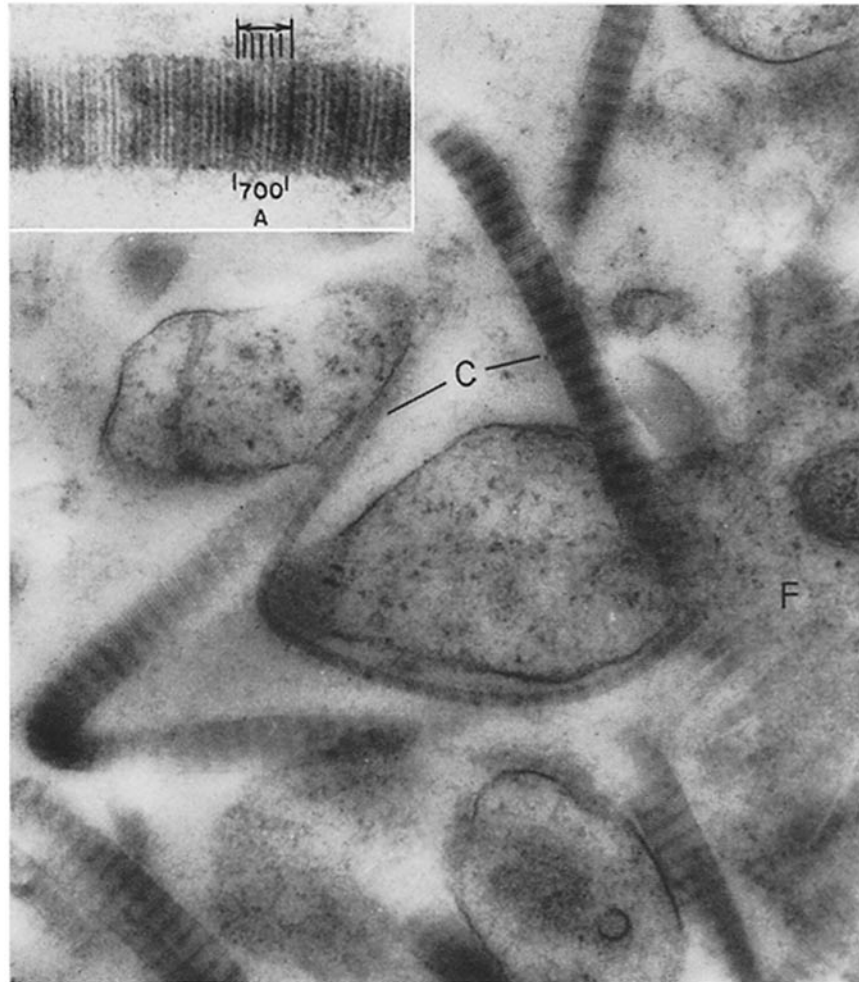
FIGURE 13

Micrograph of a section of a 14 day wound. The tip of a fibroblast (F) is cut tangentially so that collagen fibrils (c) give the appearance of streaming out of the cell. The 700 A periodicity is evident in these mature collagen fibrils, with five interperiod bands present between adjacent pairs of 700 A bands. × 36,000.

The insert (upper left) shows an enlargement of one of these fibrils, more clearly demonstrating the intraperiod banding. × 100,000.

the central part of a 56 to 70 A cylinder, and therefore capable of giving the characteristic 700 A periodicity (Fig. 14 A, B). Because of this the unit fibril width would have to be 70 A to accommodate at least 3 or 4 rows of 4 or 5 tropocollagen molecules in the center, giving a region of *ca.* 50 A width in this area which could produce the banded appearance.

The positioning of the tropocollagen molecules within the collagen fibril must be considered in three dimensions to account for all the possible arrangements of the molecules which could give rise to the structure of native collagen. In light of this, it is conceivable that the "quarter-stagger" system mentioned above could be a *quarter-stagger effect*, rather than an actual neighbor-to-nearest-neighbor one-fourth length displacement. If sufficient numbers of tropocollagen molecules were present, such an arrangement could still give the 700 A periodic banding. Assuming that the quarter-stagger arrangement is a statistical representation of a random distribution of molecules, in multiples of 700 A, producing the effect, the problem of minimum fibril size necessary to display the characteristic banding becomes a question of probability (57).

Calculations of fibril width necessary to display a 700 A banding are based upon the assumption

that a given number of tropocollagen units are arranged to give a quarter-stagger effect. If they are so arranged, the minimal filament or fibril width which could display this periodicity is approximately 100 A. Under highly optimal circumstances a width of 70 A might display this structure. Certainly, if the width were less than this, one could not expect to be able to see the structure so characteristic of collagen. The smallest fibril showing typical collagen banding observed in this study is about 100 A. Other factors inherent in the ability to resolve a periodic banding are: the staining intensity, which is related to the width of the fibril; the thickness of the tissue section in which the fibril is situated; and the resolution of the microscope.

The numerous intracytoplasmic filaments seen in the fibroblasts in the present investigation show no visible 700 A banding. From the foregoing analysis, it is evident that we are in a zone of observation in which we cannot be certain whether such banding is or is not present. It is possible that with improvement in technique some of these filaments may be shown to have a periodicity. However, below 56 A in width it is hard to conceive that a 700 A banding will be found, and therefore other means may be required to identify these filaments as collagen.
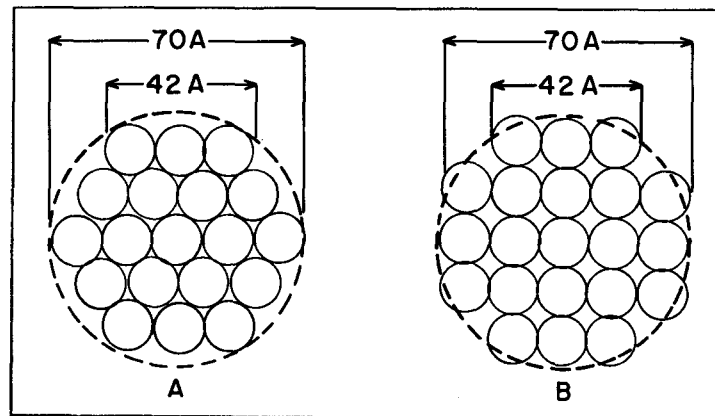

FIGURE 14

A and B represent two hypothetically possible arrangements of the tropocollagen molecules as seen in cross-section. These figures demonstrate that in a fibril of 70 A diameter only the 3 central rows of molecules would contain at least 4 tropocollagen units in a given direction, giving a region of 42 A which should be capable of displaying a 700 A periodicity. The arrangement of the molecules must be considered in three dimensions to point out both the possibilities and the difficulties inherent in determining whether a 700 A banding will be present.

Other Fibroblast Characteristics

It should be noted that, contrary to the early light microscopic findings related to collagen formation, no syncytium of the cells can be seen. All cells are distinct and separate. The component analogous to the "fibroglia fibers" seen in the light microscope by early workers (44) undoubtedly is the many fine, narrow cytoplasmic processes of the fibroblasts, which reach in many directions and approximate the processes of neighboring cells.

Enlarged mitochondria with irregular cristae are seen in a great number of fibroblasts, while those in the adjacent macrophages do not display these alterations. Lehninger (46) and Lehninger and Ray (47) have pointed to the fact that enlarged or "swollen" mitochondria probably represent a change in the functional state rather than a pathologic change within these organelles. The mitochondria are capable of a severalfold increase in volume without rupture of their membranes. He has been able to relate this change in size to the oxidation state of enzymes present in the mitochondrial membranes. Green and Hatefi (45) have related membrane loss and other changes in these organelles to the loss of activities such as oxidative phosphorylation. If changes such as these are not artifacts, further investigation into their origin should prove to be of great interest.

Little blood vessel proliferation is seen in the wounds observed in the present experiments. It is assumed that this is due to the fact that a narrow wound capable of healing by first intention was created. The wounds were kept as narrow as was feasible in order to limit the area of study in the electron microscope.

In tissues prepared for routine light microscopy, it is difficult to distinguish more than one cell type within the wound area at the later times. This contrasts with our finding that two cell types are regularly seen by electron microscopy. The first type is the fibroblast, and the second type is characterized by a paucity of endoplasmic reticulum. The latter may be either a macrophage, a fibroblast in the resting phase, or a less mature cell type with or without the potential of becoming a fibroblast. Peach et al. (59) discuss two forms of fibroblasts in their study of regenerating tendon. The two forms we describe here are two different cell types and are clearly distinct morphologically.

A great deal of effort and speculation concerning the role of the mucopolysaccharides in collagen formation and wound healing has been put forth in recent years. The presence of metachromatic material in healing wounds is a well known fact. It is now clearly established (60–62) that fibroblasts can synthesize mucopolysaccharides during wound healing and in vitro. There is no clear evidence that the mucopolysaccharides enter into collagen formation. Furthermore, collagen fibrils can be formed in vitro without the participation of mucopolysaccharides (9, 63). With the techniques of tissue preparation for electron microscopy used in these studies there is no differentiation of the mucopolysaccharides. Perhaps with appropriate fixation, and histochemistry applied to electron microscopy, a description of the disposition and changes in these substances in relation to fibroblast activity will be obtained.

We have presented here a composite view of the many changes which take place during the process of wound healing. The most striking early feature is the presence intercellularly of cytoplasmic constituents of the inflammatory cells which are subsequently ingested by the macrophages. Later the fibroblast can be clearly distinguished by the constellation of its cytoplasmic constituents. The sequence of collagen formation has been described within the 14-day period of wound healing. It is concluded that the precise manner in which the tropocollagen units are released into the extracellular environment and the relation of the cell to the polymerization of these units are questions which require further investigative effort. The fundamental question of the precise orientation of tropocollagen molecules in a three-dimensional arrangement which can give rise to the characteristic banding of collagen has been raised, and a hypothesis relevant to its answer has been made. The relation of fibril width to the visualization of the characteristic 700 A banding of collagen has been demonstrated.

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