WOUND HEALING AND COLLAGEN FORMATION

IV. Distortion of Ribosomal Patterns of Fibroblasts in Scurvy

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ABSTRACT

The changes in scorbutic wounds following the administration of ascorbic acid have been investigated using the techniques of electron microscopy, histochemistry, and autoradiography. Particular attention has been paid to the changes seen in the endoplasmic reticulum of the fibroblasts and to the identity of the extracellular filamentous material characteristic of scorbutic wounds. Seven-day-old wounds in scorbutic guinea pigs were examined prior to and from one to 72 hours following the administration of vitamin C. Fibroblasts from wounds of normal animals demonstrate a characteristic configuration of the ribosomes of the endoplasmic reticulum which is suggested to be analogous to polyribosomes described in cells synthesizing protein such as the reticulocyte. Tangential views of the membranes of the ergastoplasm show the ribosomes to be grouped in paired rows which take both straight and curved paths. This configuration is lost in scurvy and can be seen to begin to reappear as early as 4 hours after giving ascorbic acid. With increasing time, the morphology of the ribosomal aggregates approximates that seen in normal cells, so that by 24 hours their reorientation is complete. It is suggested that one of the disturbances in scurvy may relate to an alteration either in messenger RNA, in the ability of the ribosomes to relate to the messenger, or in the membranes of the ergastoplasm. In addition, the lack of formation of hydroxyamino acids necessary for completing collagen synthesis may be related to the architecture of the ribosomal aggregates. Extracellular collagen fibrils appear concomitant with the restoration of ribosomal and ergastoplasmic morphology as early as 12 hours after administration of ascorbic acid, with complete disappearance of the scorbutic extracellular material within 24 hours. Observations of this scorbutic material do not support the concept that it is a collagen precursor.

INTRODUCTION

Ascorbic acid has a critical role in both the formation and maintenance of collagen in healing wounds of man and guinea pigs, as well as in the prevention of hemorrhage from the vascular components of connective tissue (4–6, 20–22). Scurvy has been known for over two hundred years in man (1, 2). The studies of Aschoff and Koch (3), Höjer (4), and Wolbach and Howe (5, 6) describing the changes seen in this disease in a variety of connective tissues are now classical. The latter authors carefully described the lack of collagen formation in dentin, bone, and healing

wounds, during the ascorbic acid–deficient state. They described the appearance of a substance, lacking the ordinary staining characteristics of collagen, in the interstitial spaces of these tissues. After the administration of ascorbic acid, they observed the prompt appearance of argyrophilic fibers, followed shortly by fibers having the typical staining characteristics of collagen (5, 6, 25).

The studies with the light microscope left the following questions unanswered:

1. What is the nature of the cellular alteration resulting from scurvy?
2. What is the nature of the material appearing in the extracellular spaces and adjacent to the abnormal fibroblasts during their proliferation in scorbutic wounds? Where does this material come from?; is it elaborated by the fibroblasts, or is it produced elsewhere?
3. Do the argyrophilic fibers and the collagen, found very soon after ascorbic acid administration, come from material newly elaborated by the fibroblasts, or are these formed from material pre-existing in the interstitial space?

In our previous investigations (7–9), the observations of the light microscopist were extended, and certain aspects of the cellular alterations were presented. The aim of the present studies is to further characterize the deviations seen in the scorbutic state from those observed in the normal state during the process of wound healing, and to answer, if possible, some of the remaining questions. The principal previous findings (7–9) established:

1. Several aberrations in the appearance of the fibroblasts in the scorbutic state. These consisted of (*a*) an altered endoplasmic reticulum which appeared in the form of rounded structures, rather than long continuous canaliculi, and (*b*) large accumulations of intracytoplasmic lipid.
2. The appearance in the extracellular spaces of a fine, fibrillar material in scurvy which lacks the characteristic banding of collagen. The material was found by *in situ* histochemical methods to be low in tryptophan, and was intermixed with strands of fibrin present in these wounds. It was concluded that the morphologic findings in scurvy were consistent with an alteration in protein synthesis by the scorbutic fibroblasts.
3. The autoradiographic studies with tritiated proline demonstrated a rapid passage of the label into normal cells with its subsequent release, and appearance over the extracellular collagen fibrils. Several differences were noted in the scorbutic wounds. The uptake of proline was somewhat slower than in normal cells, possibly due to the alteration in the vasculature of these wounds. In addition, the scorbutic fibroblasts took up the same relative amount of label as the normal cells, but lost it at a slower rate. The appearance of the label in the extracellular fibrillar material in wounds of scorbutic animals also occurred more slowly, and continued to increase in amount to the end of the experiments. In contrast to this, the label in the collagen of normal animals leveled off after 24 hours, and began to decrease, presumably due to dilution, after the 3rd day. This continuing increase of label in the scorbutic material could possibly represent an influx of labeled protein from the blood, although the reasons for this rise are not yet clear.

The present investigation was designed to examine the wounds following the administration of ascorbic acid and the restoration of a normal diet. Of particular interest is the advent of collagen formation and its association with the changes in appearance of the various cellular organelles associated with protein synthesis. In addition, it was hoped that further examination by autoradiography and enzyme histochemistry of these wounds would shed light on the identity of the filamentous extracellular material in the scorbutic wounds.

MATERIALS AND METHODS

Tissue Preparation for Light Microscopy

Female guinea pigs weighing approximately 300 grams were placed on a scorbutigenic diet (Nutritional Biochemicals, Cleveland) for 14 days. All of the operative procedures were performed under ether anesthesia. Circular wounds were created in the dorsal skin, as described previously (7), and allowed to heal for 10 days. A wound was removed from each animal on the 10th day and ascorbic acid (100 mg) was administered intraperitoneally. The animals were then placed on a diet containing adequate ascorbic acid. The remaining wounds were removed at 24-hour intervals for 5 days following vitamin C administration.

The wounds were fixed in neutral buffered formalin and embedded in paraffin. Sections were stained

with hematoxylin and eosin, the Van Gieson stain, Mallory's connective tissue stain, Wilder's reticulin stain, phosphotungstic acid–hematoxylin, and Weigert's stain for fibrin.

Histochemical Techniques

Cross-sectional blocks of scorbutic wounds were removed and quick frozen in liquid nitrogen-cooled isopentane. Six-micron sections were cut from this material in a Pearce cryostat. The sections were treated in the following way:

1. Sections from each animal were examined for the presence of tryptophan by the Adams' indole method (31).
2. A group of sections from each animal were incubated in collagenase (Worthington Biochemical Corp., Freehold, New Jersey) (pH 7.0) for 30 minutes, and for 1, 2, and 4 hours.
3. Another group of sections were incubated in crystalline trypsin (Armour Pharmaceuticals, Kankakee, Illinois) (pH 8.0) for 30 minutes, and for 1, 2, and 4 hours.

Several groups of blocks of wound tissue were fixed in cold acetone, or alcohol (for 12 hours), or 70 per cent methanol for 3 hours. These were then embedded in paraffin, sectioned, and exposed to either collagenase or trypsin.

All sections were stained with hematoxylin and eosin, Van Gieson, or by the Adams' indole method.

Control sections for each of the above were incubated in the appropriate buffer, in a fashion similar to that of the enzyme incubations, and subsequently stained.

Tissue Preparation for Electron Microscopy

With the animal under ether anesthesia (8), linear incisions, approximately 1 cm long, were made in the dorsal skin of a separate group of 12 female guinea pigs after these animals had been fed for 14 days on the scorbutigenic diet. A wound was removed, as previously described (7), prior to ascorbic acid administration. Each animal was then given 100 mg of ascorbic acid intraperitoneally, placed on a normal diet, and wounds were removed at 4-hour intervals for the first 24 hours and at 24-hour intervals for the next 3 days.

The wounds were cut into transverse slices 1 to 2 mm thick, as described previously (7), and were then fixed. Sections from each of 4 to 5 blocks were examined from the central portion of each wound. These tissues were fixed in 2 per cent osmium tetroxide buffered with *s*-collidine (10), fixed again in neutral buffered formalin, and embedded in Epon 812 (11). Adjacent 1-micron sections were stained with Azure II, Methylene Blue (30) and used for orientation and specimen trimming so that the thin sections represented the center of each wound. Hence, all of the micrographs taken were of the wound contents. At least 50 sections were examined of wounds from *each* of the 5 time periods.

In an effort to resolve the problem of sampling, a large number of micrographs (ranging from 20 to 50) were taken randomly of each section. Each micrograph contained from 1 to 5 cells, with an average of 2 cells; consequently, no less than 2,000 cells, and often many more, were examined from each of the five time periods. The sections were stained with either uranyl acetate, lead hydroxide (12), or a combination of uranyl acetate followed by lead hydroxide. The tissues were examined with an RCA-EMU 3E or 3G electron microscope. The description of fibroblasts from non-deficient animals represents a much larger number of observations from previous and present experiments (7–9, 51).

Techniques Used for Autoradiography

A third group of four female guinea pigs was placed on a scorbutigenic diet for 14 days. Each animal was wounded under anesthesia by making seven linear incisions in the dorsal skin. The wounds were allowed to heal for 7 days, and each animal was then given proline-3,4-H^3 (New England Nuclear Corp., Boston) (12 μc/gm body weight). A wound was removed from each animal at 6 days after proline-H^3 administration. After removal of this wound, each guinea pig was given 100 mg of ascorbic acid intraperitoneally and placed on a normal diet. Wounds were then removed at 4, 12, 24, 36, 48, and 72 hours following vitamin C administration. These tissues were fixed and embedded as for electron microscopy, sectioned at 1-micron, and prepared for autoradiography using NTB-3 nuclear track emulsion (Eastman) as previously described (9).

The autoradiographs were developed after 6 weeks' exposure, and random photomicrographs were taken of sections from three different blocks of tissue from each wound. The micrographs were enlarged and the numbers of grains were counted per unit area of cells, collagen, filamentous material, erythrocytes, and debris, as previously described (9). These counts were then corrected for background.

Nutritional Behavior of the Animals

Each animal was weighed every other day. Earlier experiments (8, 9, 35, 54) demonstrated that regular examination of weight, prior to the period of extreme weight loss from inanition, is a reliable indication of food intake in these animals. In addition, previous pair-feeding experiments (unpublished data) demonstrated that the weights of pair-fed animals are similar to those of the scorbutic animals during the experimental period. Gross also observed this and noted, in addition, that pair-feeding indicates that the weight loss in the latter stages of scurvy is not due to inanition

alone (35). As in the previous experiments in scurvy (8, 9), the animals gained weight on the scorbutigenic diet for the first 12 days. Their weights subsequently leveled off and remained stable for approximately 4 to 6 days, after which time they began to decrease. The weight curves are very similar to those observed by Gross (35) in his studies of scurvy following ascorbic acid administration. After being given ascorbic acid, all of the animals returned to a normal weight-gain pattern. Due to the trauma of wounding, each animal characteristically loses weight for a day and then proceeds to gain weight.

At the end of the experiments, each animal was sacrificed and the molars and incisors were examined for evidence of scorbutic change in areas in the odontoblasts of the pulp. Changes characteristic of scurvy followed by healing were observed.

LIGHT MICROSCOPE OBSERVATIONS

The sequence of events observed in the light microscope is as follows: Prior to ascorbic acid administration, the extracellular spaces contain material which is poorly structured (8). This material does not stain for reticulin or collagen with either the Van Gieson or Mallory connective tissue stains. This configuration appears essentially unchanged up to 24 hours following the administration of ascorbic acid; however, by 24 hours some argyrophylic fibers appear. These fibers are narrow at first and become coarse and wavy by 48 hours. Most of them appear to lie in planes parallel to the borders of adjacent cells. Twenty-four hours after ascorbic acid administration, there is material present between the cells that is faintly positive with the Van Gieson stain. Within 48 hours, however, strongly Van Gieson-positive fibers appear. The same sequence is observed with the Mallory connective tissue stain. These findings parallel those already described by Wolbach and Howe (5). They re-emphasize the fact that prior to 24 hours after ascorbic acid administration it is difficult to find evidence for collagen formation using these techniques.

ELECTRON MICROSCOPE OBSERVATIONS

The electron microscopic observations cited below are the result of sampling more than 250 tissue blocks from all of the animals, followed by examination of randomly taken electron micrographs representing more than 2,000 cells from each time period. The observations listed are representative for all of the tissues from the controls, scorbutic and recovery wounds examined after the various time intervals.

In addition, a comprehensive review was made of electron micrographic data from previous wound-healing experiments from normal and scorbutic animals (7–9, 51). The observations of the morphology of the cells in the present study are in agreement with those from these earlier investigations.

One group of animals, not on a deficient diet, was pair-fed to a group of scorbutic animals. The pattern of ribosomes in fibroblasts from the wounds of the pair-fed animals were identical in appearance to those from wounds of animals fed a normal diet *ad libitum*.

Wounds Prior to the Administration of Ascorbic Acid

Many of the features of wounds from scorbutic animals have been described previously (8, 38). We should like to emphasize three specific observations which are a general feature of all of the changes in the fibroblasts in scurvy. These are:

(*a*) The rounded up, somewhat dilated, irregular cisternae of the endoplasmic reticulum, and decreased amount of rough-surfaced endoplasmic reticulum.

(*b*) A disappearance, in scurvy, of the characteristic orientation of the ribosomes attached to the cisternal membranes of the endoplasmic reticulum seen in normal fibroblasts.

(*c*) An increase in the number of free cytoplasmic ribosomes in scorbutic fibroblasts.

The first of these, the rounded, separated profiles of the endoplasmic reticulum, was noted earlier (8, 38). The characteristic appearance of the cells can be seen in Fig. 1. It should be noted that most of the membranes of the cisternae of the endoplasmic reticulum contain a full complement of ribosomes and there are few empty sites apparent on the membranes.

The second change in scurvy is apparent in the configuration of the attached particles lining the rounded-up cisternae. The orientation of the ribosomes on the endoplasmic reticulum can be seen in tangential sections of the cisternal membranes that provide an *en face* view. In guinea pigs fed adequate amounts of ascorbic acid, the ribosomes of the wound fibroblasts present a characteristic and possibly specific configuration. They appear to be grouped in rows of pairs, which often curve

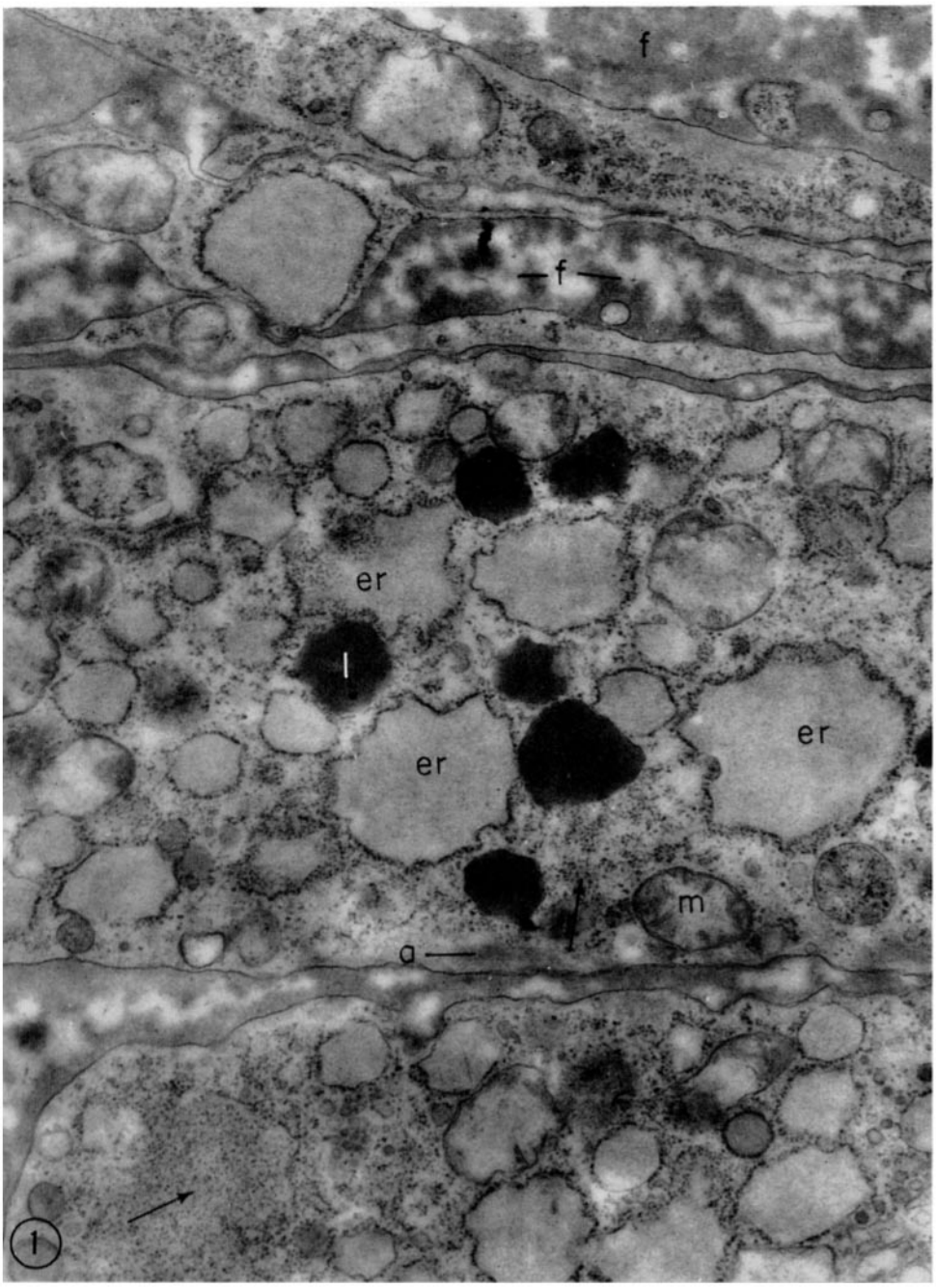


FIGURE 1 An electron micrograph from a portion of a 7-day-old scorbutic wound. The cisternae of endoplasmic reticulum (*er*) appear as rounded, separate profiles which contain somewhat dense, amorphous material. The surface of these rounded cisternae often appear ruffled and irregular. Lipid deposits (*l*) are evident as well as large mitochondria (*m*). Numerous free ribosomes (arrows) and peripheral cytoplasmic, filamentous aggregates (*a*) can also be seen. The extracellular spaces contain the non-banded filamentous material (*f*) characteristic of scorbutic wounds. × 21,000.

and present spiral or rosette forms (Figs. 2 *a* and 2 *b*). In normal cells there are very few free ribosomes and most of the attached particles can be seen to have this orientation whenever the membranes are tangentially sectioned. Such *en face* views are most commonly seen in regions where the endoplasmic reticulum approximates the Golgi zone. A marked change is apparent in this configuration in scorbutic fibroblasts. Instead of being regularly arranged in rows of two, the ribosomes present a different configuration and appear to be randomly distributed on the surface of the cisternae (Fig. 2*c*).

Thirdly, numerous unattached ribosomes can be seen to be distributed throughout the cytoplasmic matrix of the scorbutic fibroblasts (Fig. 1). Some of them appear to be grouped in clusters, although the largest proportion are randomly dispersed in the cytoplasm. Fewer free cytoplasmic ribosomes appear to be present in the fibroblasts from normal animals.

Therefore, in scurvy, the ribosomes are altered not only in that numerous free particles are present in the cytoplasm of scorbutic fibroblasts, but also in that those that are attached to the membranes of the endoplasmic reticulum have lost the pattern so characteristic of non-deficient fibroblasts.

Intracytoplasmic lipid deposits and the peripheral filamentous aggregates in the scorbutic cells have been reported previously (8).

The extracellular material present in the scorbutic wounds is generally homogeneous, consisting of broad interlacing bundles of fine filaments closely packed together. Occasional bundles of fibrin, recognizable by its characteristic periodicity, can be seen intermeshed within this material, as well as occasional bits of cell debris.

An additional finding of interest in wounds of scorbutic animals is that the rough endoplasmic reticulum of the macrophages does not appear to be altered in contrast to that of the fibroblasts.

4 Hours after Ascorbic Acid

Four hours after ascorbic acid administration, the wounds are similar to those seen in scurvy. The fibroblasts contain numerous profiles of rounded cisternae of endoplasmic reticulum which do not appear to be interconnected, at least within the plane of section. However, in a few regions, paired rows of ribosomes in curved shapes can already be seen on the cisternae of the endoplasmic reticulum (Fig. 3, arrows). Lipid deposits previously described are also present in these cells. The filamentous aggregates located in the periphery of the cytoplasm are present in these cells just as they appear in the fibroblasts of both normal and scorbutic animals.

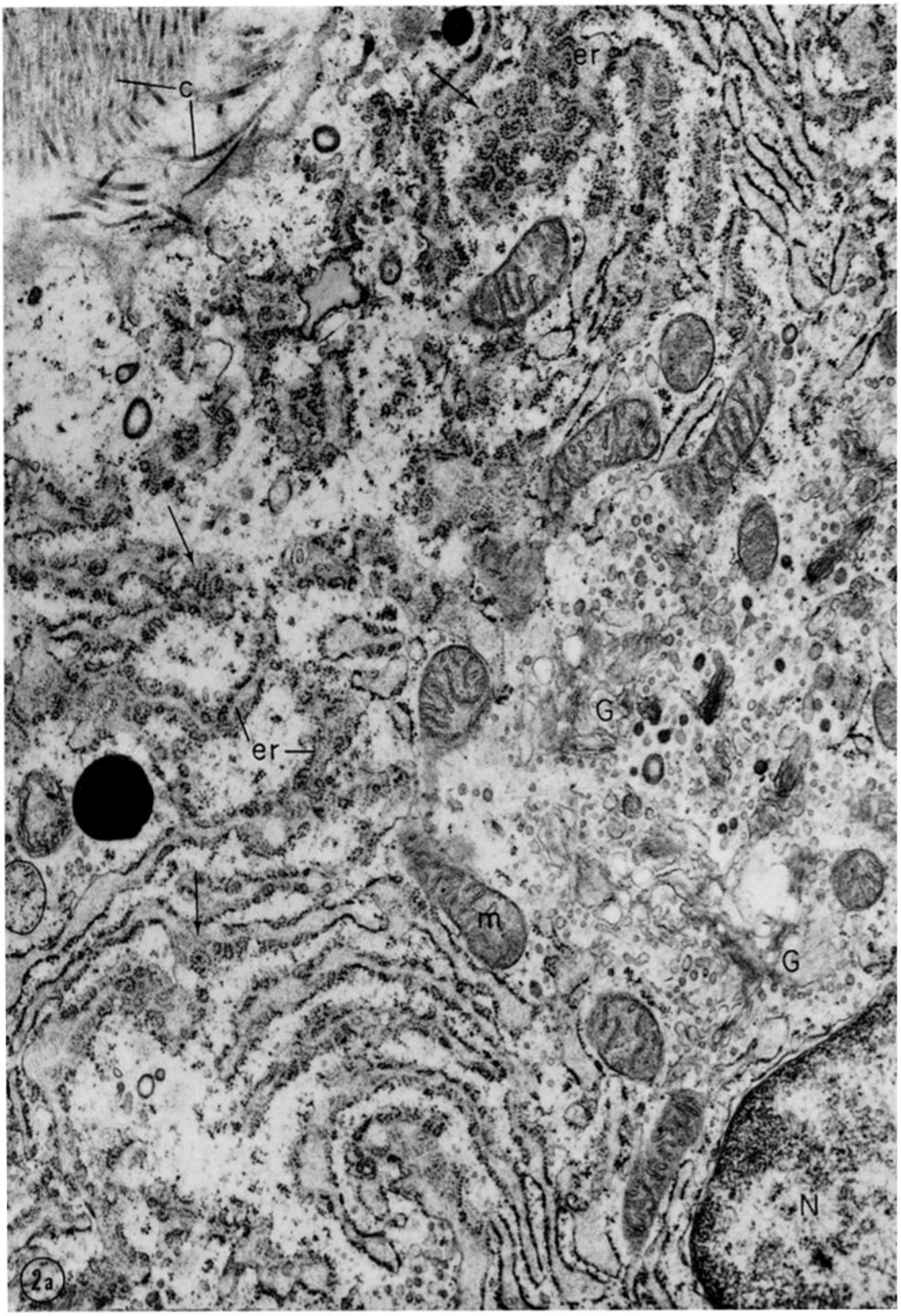
The intercellular spaces contain large, broad, interlacing bundles of material composed of what appear to be fine filaments. No banding can be observed in either the bundles or the filaments after staining with either uranyl acetate or lead hydroxide, except in those few areas in which fibrin can again be identified. Extravasated erythrocytes and small bits of material resembling cell debris are intermeshed within this fibrillar material.

8 Hours after Ascorbic Acid

By 8 hours, many of the cisternae of the endoplasmic reticulum appear dilated (Fig. 4) and somewhat more interconnected than at 4 hours. Many free ribosomes are still present in the cytoplasm of these cells, and numerous intracytoplasmic lipid deposits are present. At this time, the reorientation of the ribosomes attached to the endoplasmic reticulum of the fibroblasts appears to be more advanced than earlier (Fig. 4).

The mitochondria are typical of those seen in fibroblasts, and peripheral filamentous aggregates are present in these cells. In addition, the Golgi complex appears to be larger and more extensive

FIGURE 2 *b* This represents part of a fibroblast from a 7-day wound in a non-deficient animal. The nucleus (*N*), mitochondria (*m*), and Golgi complex (*G*) of this cell are apparent. A characteristic feature of this cell is the extensive, well developed rough endoplasmic reticulum (*er*) that is arranged in the form of numerous flat sacs often interconnecting with each other. When the membranes of the ergastoplasm are sectioned tangentially, the ribosomes attached to their surface appear to be arranged in characteristic patterns (arrows), not visible when the membranes are sectioned normally. A higher magnification of such a region is presented in Fig. 2 *b*. × 20,000.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than that seen in scorbutic cells prior to treatment.

The appearance of the material in the extracellular spaces varies from area to area. In most regions the characteristic scorbutic, non-banded fibrils can be seen (Fig. 4). However, there are regions in which extracellular condensations of material can be seen adjacent to cell surfaces. In addition, a fine filamentous material of lower density can be seen to be intermixed with the material typical of scorbutic wounds.

12 Hours after Ascorbic Acid

At this time period, changes can be seen in both the cells and the extracellular regions. The numbers of free cytoplasmic ribosomes has decreased, and in some parts of many of the cells tangential views of the cisternal membranes further demonstrate a tendency to form rows of paired ribosomes (Figs. 5, 6). Much less intracytoplasmic lipid is present in many of the cells. The profiles of endoplasmic reticulum appear longer, narrower, and more interconnected than previously (Figs. 5, 6). The Golgi regions of the fibroblasts are prominent and their vesicles and cisternae appear to be dilated. Numerous vesicles of varying size can be seen to line some cell surfaces at this time.

The area between the cells now consists of fibrils, some of which are clearly collagen, as evidenced by a 700-A periodic banding, intermixed with a much larger mass of material consisting of fine filaments which display no banding (Figs. 5, 6).

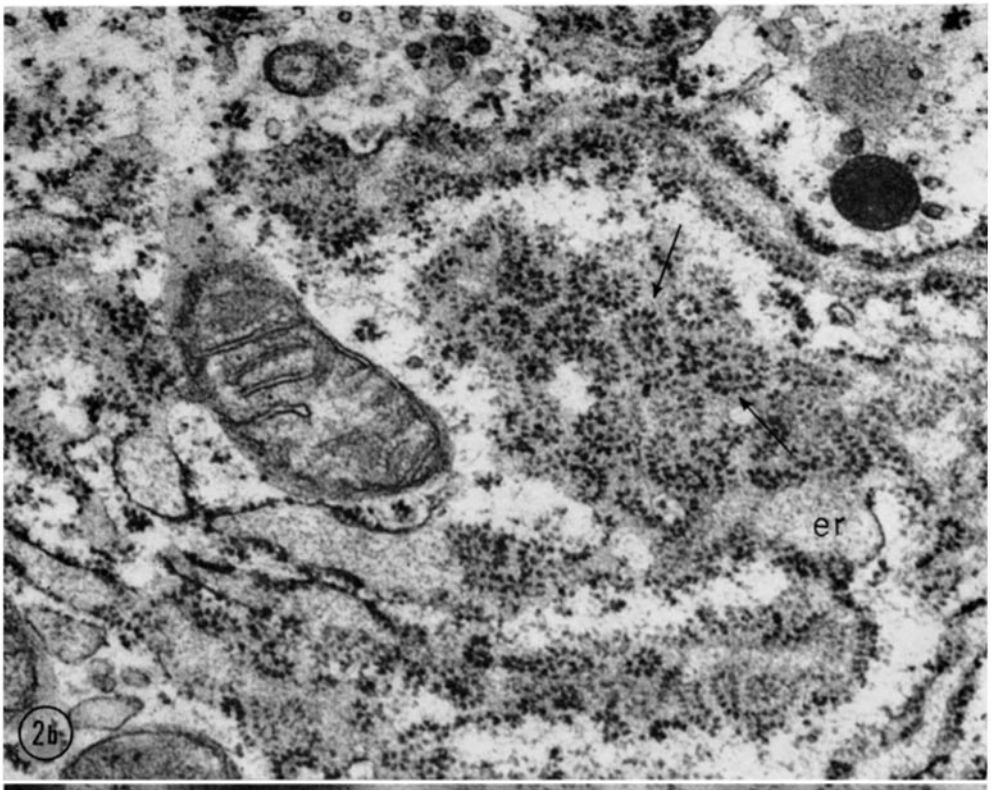
24 Hours after Ascorbic Acid

By 24 hours after the administration of ascorbic acid, the wounds appear very similar to those seen in normal animals. The extracellular spaces are filled with collagen fibrils. The non-banded filamentous material seen in scurvy has completely disappeared (Fig. 7). The fibroblasts now appear very much like those seen in non-deficient animals (7). They contain an abundant endoplasmic reticulum, large mitochondria, and a prominent Golgi region. The attached ribosomes demonstrate an arrangement similar to that seen in the non-scorbutic cells (Fig. 8). Tangential sections show rows of pairs of ribosomes lying upon the membranes of the endoplasmic reticulum in both straight and curved profiles (Fig. 8). A relatively small number of free ribosomes, grouped in clusters or rosettes, remain in the cytoplasm of these cells. Many of the recovering fibroblasts contain numerous peripheral vesicles as well.

A common additional observation made in a large number of micrographs was noted in relation to the endoplasmic reticulum of the cells of the 24-hour ascorbic acid–treated animals. Whenever the membranes of the cisternae approach the plasma membrane they appear to be devoid of ribosomes. Numerous profiles of cisternal membranes have been observed so close to the plasma membrane that they appear to be in contact (Fig. 9). Direct continuity of the cisternal cavity with the extracellular milieu has, however, never been demonstrated in any of this material. Numerous vesicles can also be seen between the

FIGURE 2 *b* This micrograph displays part of the fibroblast from a 7-day wound seen in a normal animal in Fig. 2 *a*. The region of the cell depicted shows part of a mitochondrion, as well as several profiles of rough endoplasmic reticulum (*er*). Where the cisternae are sectioned normally, a single layer of ribosomes may be seen attached to the membranes. However, there are many regions (arrows) where the ergastoplasmic membranes have been grazed tangentially, presenting an *en face* view of the orientation of the ribosomes attached to the membranes. Here the membrane appears as an increased area of density around the ribosomes. The ribosomes are arranged in characteristic curved and spiral patterns, some of which are in double rows. In some regions (arrows) a fine thread appears to connect these ribosomes. × 52,000.

FIGURE 2 *c* A micrograph from parts of two cells from a 7-day-old scorbutic wound. The rounded cisternae of endoplasmic reticulum are irregular in shape, and tangential sections of the membranes provide a view of ribosomal orientation. Here the ribosomes have no characteristic configuration (arrows), but appear to be randomly dispersed on the membranes. Lipid droplets and free cytoplasmic ribosomes are also apparent. × 34,000.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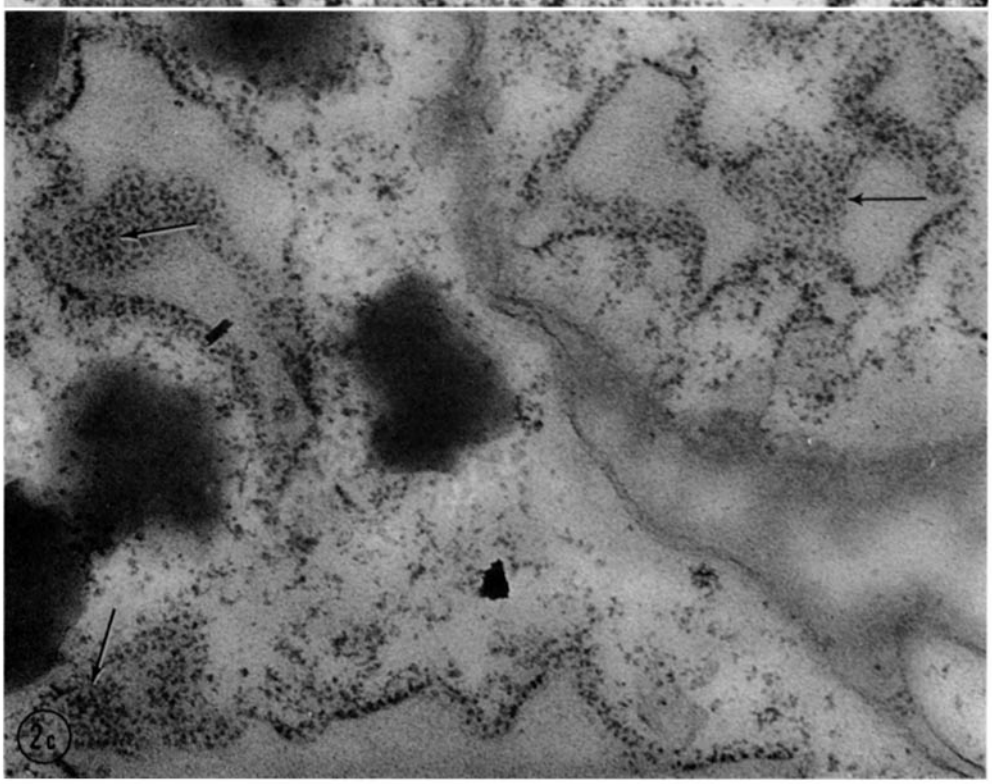
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endoplasmic reticulum and the plasma membrane of the fibroblasts.

Extracellular Changes

Observations of the extracellular, filamentous, non-banded material present in scorbutic wounds demonstrate its relatively rapid disappearance following ascorbic acid administration. Within 8 hours, non-banded filaments of lower density are present between the filaments of the scorbutic material (Fig. 4). Within 12 hours, collagen fibrils are present both adjacent to cells and intermixed with the scorbutic material (Fig. 5, 6). By 24 hours, the material present during scurvy is no longer observable, and it has been replaced by mature collagen fibrils (Fig. 7). No evidence of phagocytosis of the scorbutic material was observed at any time during the experimental observations, although phagocytosis of erythrocytes, and ferritin-containing vesicles, are clearly present in the macrophages during this interval.

48 Hours after Ascorbic Acid

As the wounds grow older, they take on the characteristics previously described for maturing wounds (7) in normal animals. Few free ribosomes are present in the cells, and those lining the endoplasmic reticulum again have their characteristic configuration. The cisternae of the endoplasmic reticulum appear as elongated, interconnected profiles, and the lipid deposits have completely disappeared from the cells. By this time, most of the extracellular regions are filled with collagen fibrils (Fig. 10).

Histochemical Observations

Examination for the presence of tryptophan in these wounds, prior to and following the administration of ascorbic acid, demonstrated a positive reaction in rare, isolated, small foci. These foci stained positively for fibrin with phosphotungstic acid–hematoxylin and the Weigert's stain. The bulk of the extracellular material gave none of these reactions.

Incubation of both frozen and chemically fixed sections, with collagenase, trypsin, or buffer, demonstrated that collagenase removed both fine and coarse collagen fibers from the adjacent wound margins. Trypsin acted on the collagen fibers in the wound edges to a lesser extent but in the same manner as did collagenase. Trypsin was also found to remove the sharply eosinophilic, tryptophan-negative extracellular material present in the scorbutic wounds, whereas collagenase did not.

The buffer alone had no effect on the collagen or the scorbutic extracellular material in any of the tissues examined.

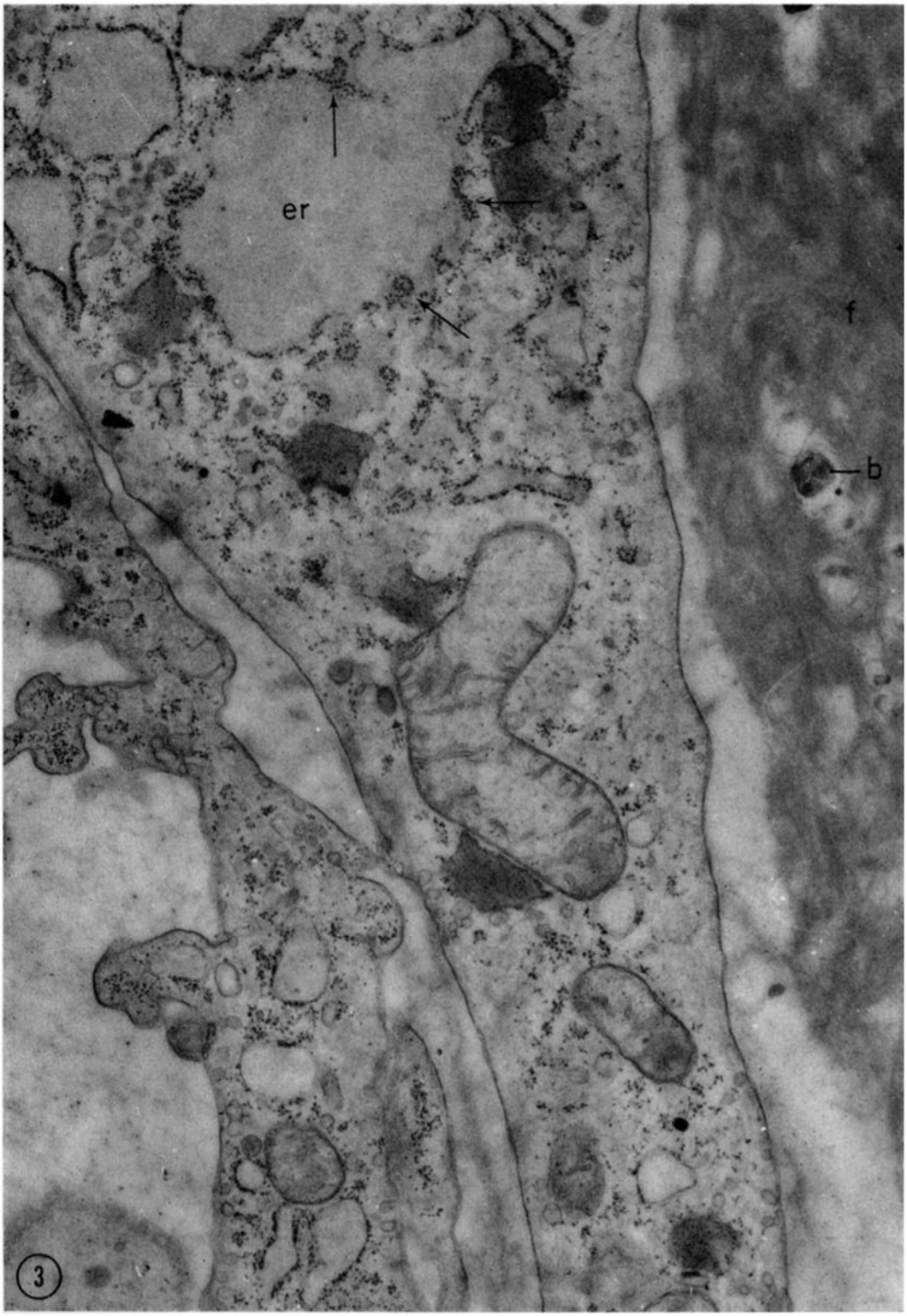
Autoradiographic Observations

The results of grain counts on the autoradiographs showed no demonstrable change in the amount of label in cells or in extracellular material at any of the time periods examined. Limitations of the autoradiographic method, as used here, preclude knowing whether any shift in isotope is occurring either from the cells to the extracellular material or from the serum to these.

DISCUSSION

Ascorbic acid deficiency presents a classical pathologic alteration of the connective tissues with a concomitant depression in the formation of a specific protein, collagen (5, 6, 17, 18, 20, 21, 23, 24, 35, 54). The present observations further characterize the fine structure alterations in fibroblasts, during scurvy, and the reconstitution of the normal ultrastructure of the fibroblast following administration of vitamin C. The reappearance of the non-scorbutic configuration of

FIGURE 3 This electron micrograph contains parts of two fibroblasts from a wound 4 hours after ascorbic acid administration. The extracellular filamentous material (*f*) appears similar to that seen prior to ascorbic acid treatment. An occasional body (*b*), possibly cell debris, can be seen intermixed in this material. The fibroblast has the appearance of a typical scorbutic cell, except that a few areas display a tendency for the ribosomes of the endoplasmic reticulum (*er*) to aggregate in a fashion similar to that seen in cells from normal animals (arrows). × 24,000.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the cell organelles is associated with a relatively rapid reappearance of mature collagen fibrils within the extracellular spaces.

Sampling

To deal with the problem of sampling is particularly important in studies utilizing the electron microscope. Because of this, we have attempted to examine a sufficiently large number of specimens. This consisted of 2,000 cells from each time period, drawn from a population of 4 blocks from each wound. These represent 12 wounds, each from a separate animal, for each time period. Within each time period examined, the appearance of both the wounds and the cells was consistent. In addition, a large amount of data from earlier studies (7–9, 51), representing both normal and scorbutic guinea pigs, was reexamined to determine similarities and differences with respect to the observations in the present series. In light of this, we feel confident that the present results represent an adequate sampling of the wounds during the course of the experiments.

Cells

Changes in the appearance of the endoplasmic reticulum of cells in other situations when protein synthesis is suppressed have been reported for various forms of liver injury (39, 43, 46–50). Smuckler, Iseri, and Benditt (39) have described in detail the changes in the liver occurring in carbon tetrachloride poisoning, and related these to the striking defect in protein synthesis occurring with this toxic injury. The principal, earliest, and most widespread change resulting from carbon tetrachloride poisoning is a loss and random scattering of the ribosomes from the cisternae of the endoplasmic reticulum. Dilation of the cisternae appears somewhat later and reaches an extreme degree only in some cells (39, 43). The scorbutic fibroblasts appear similar to the damaged hepatic cells, insofar as the occurrence of dilated, rounded cisternae of ergastoplasm, and increase in free cytoplasmic ribosomes are concerned. However, during scurvy the dilated ergastoplasmic membranes which remain, generally retain a full complement of ribosomes whose distribution on the membranes is altered.

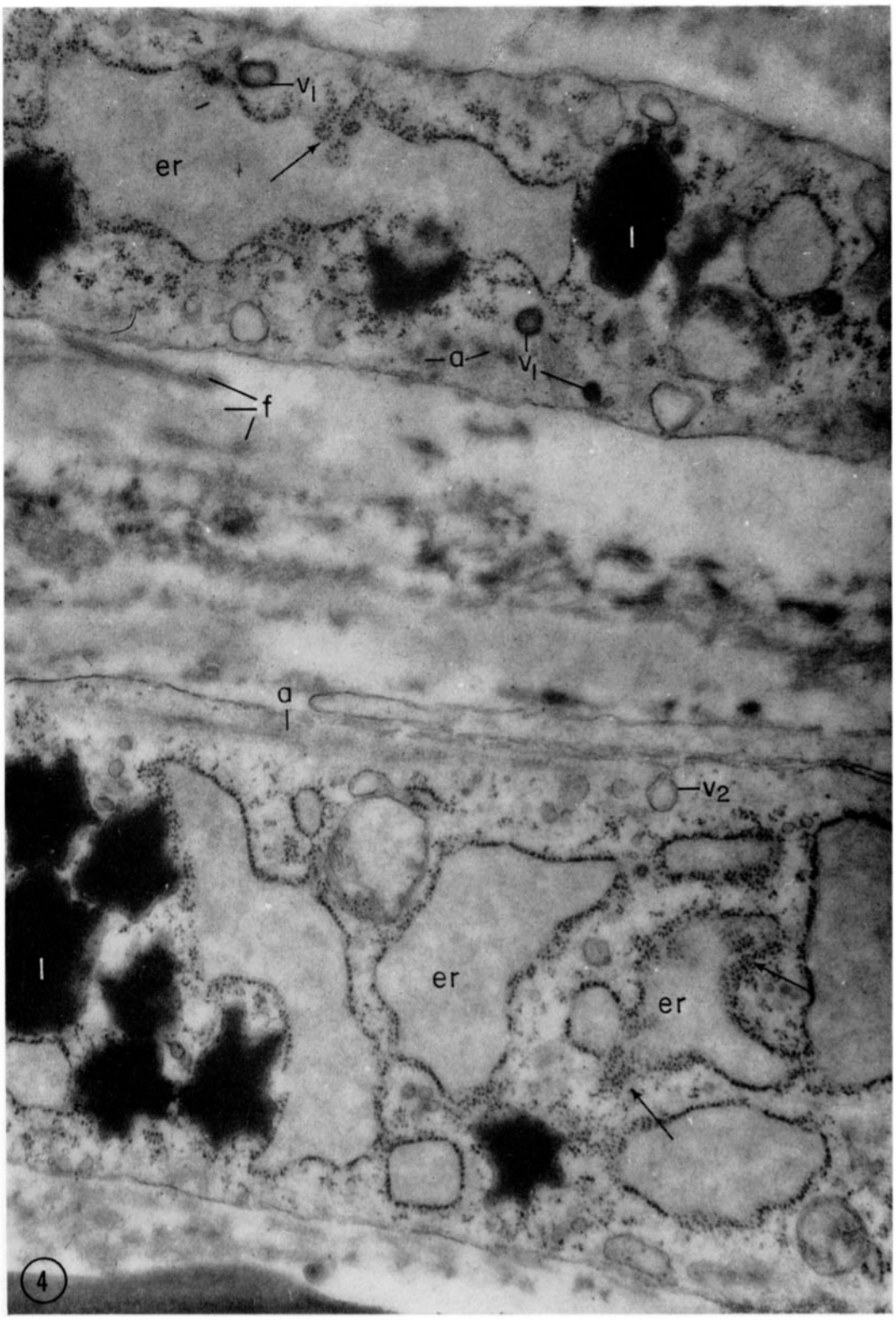
Configuration of Attached Ribosomes

An important finding relates to the specific configuration of the ribosomes on the membranes of the endoplasmic reticulum of fibroblasts from non-deficient animals. Observations of a large number of wounds from normal animals show that the ribosomes of fibroblasts take the form of parallel rows of particles which characteristically take curved forms after following a relatively short linear course (Figs. 2 *a* and 2 *b*). The same observation of ribosomal patterns was made earlier by Palade (53) in an examination of the endoplasmic reticulum of the fibroblast. Such a ribosomal orientation could be interpreted as representing *polyribosomes* (40–42, 45, 55, 56).

Warner *et al.* (42) introduced the concept, in relation to hemoglobin synthesis in reticulocytes, that ribosomes are characteristically grouped and possibly bound by messenger RNA (44), which determines the protein to be synthesized. Marks *et al.* (40) have presented further evidence which supports this concept for the reticulocytes. Staehelin *et al.* (45) gave evidence in HeLa cells in support of this idea, and recently Goodman and Rich (41), Rich *et al.* (55), and Hardesty *et al.* (56) have presented data consistent with the idea that the ribosomes move along a strand of messenger RNA and complete specific peptide or protein synthesis upon arriving at the terminal end of the strand.

As we observe above, the characteristic geometry

FIGURE 4 A micrograph of part of a wound from a scorbutic animal 8 hours after the administration of ascorbic acid. Here portions of two fibroblasts with dilated cisternae (*er*), lipid deposits (*l*), peripheral filamentous aggregates (*a*), and many vesicles can be seen. Several of these vesicles appear to have a concentric dense zone just adjacent to their delineating membrane (v_1), whereas others (v_2) do not. The extracellular filaments (*f*) appear to be of two different densities, in contrast to those in scorbutic wounds. Tangential sections of the ergastoplasmic membranes show a return to the paired-row configuration seen in normal cells (arrows). × 24,000.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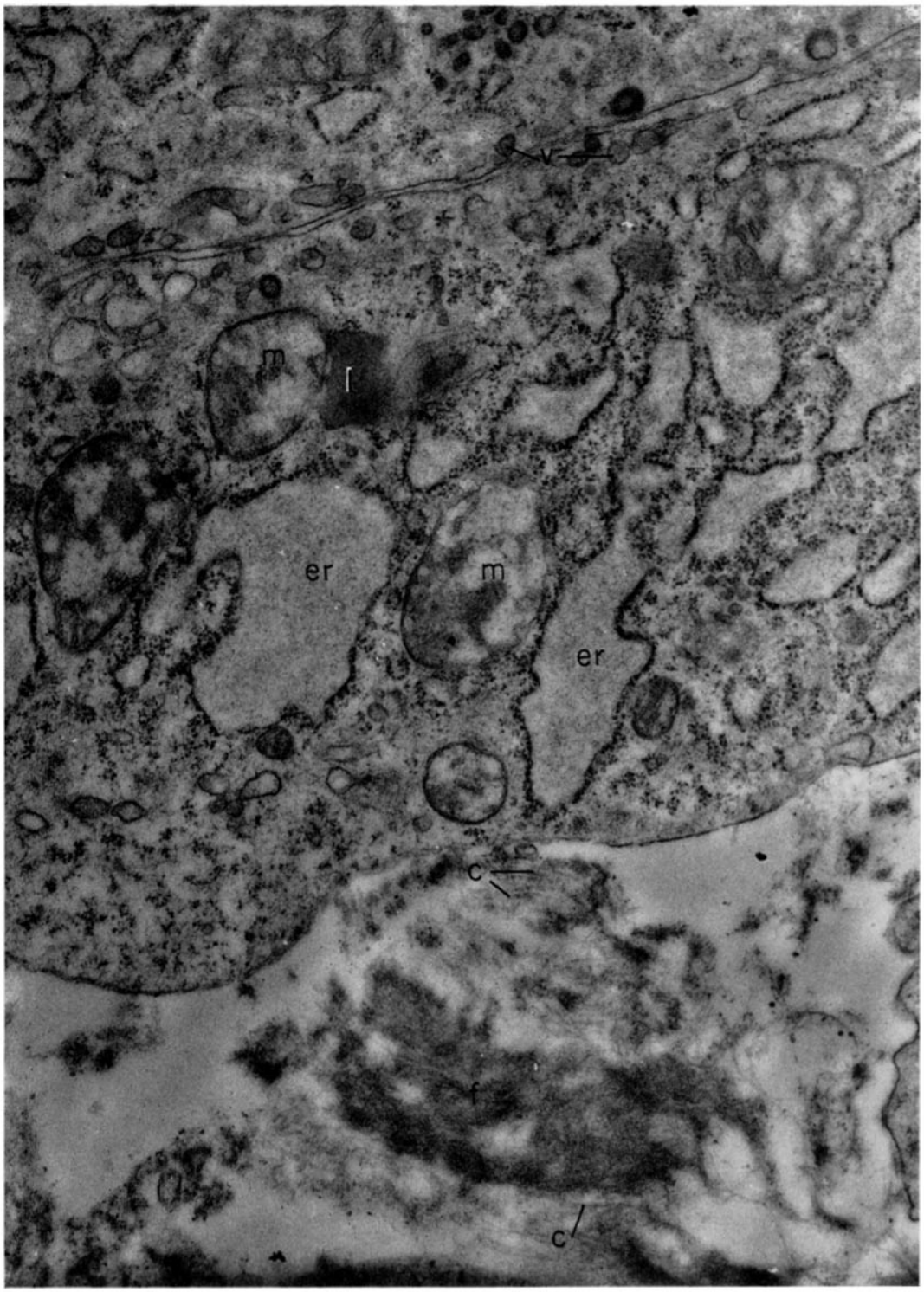


FIGURE 5 An electron micrograph of an area from a wound 12 hours after ascorbic acid administration. At this time, numerous collagen fibrils (*c*) can be seen to be dispersed among non-banded filaments (*f*) in the extracellular spaces. Parts of two fibroblasts with their mitochondria (*m*), endoplasmic reticulum (*er*), a lipid deposit (*l*), and numerous vesicles (*v*) can be seen. The ribosomes of the ergastoplasm of these two cells display an even greater tendency to the orientation characteristic of non-deficient cells than was present at 4 or 8 hours. × 28,000.

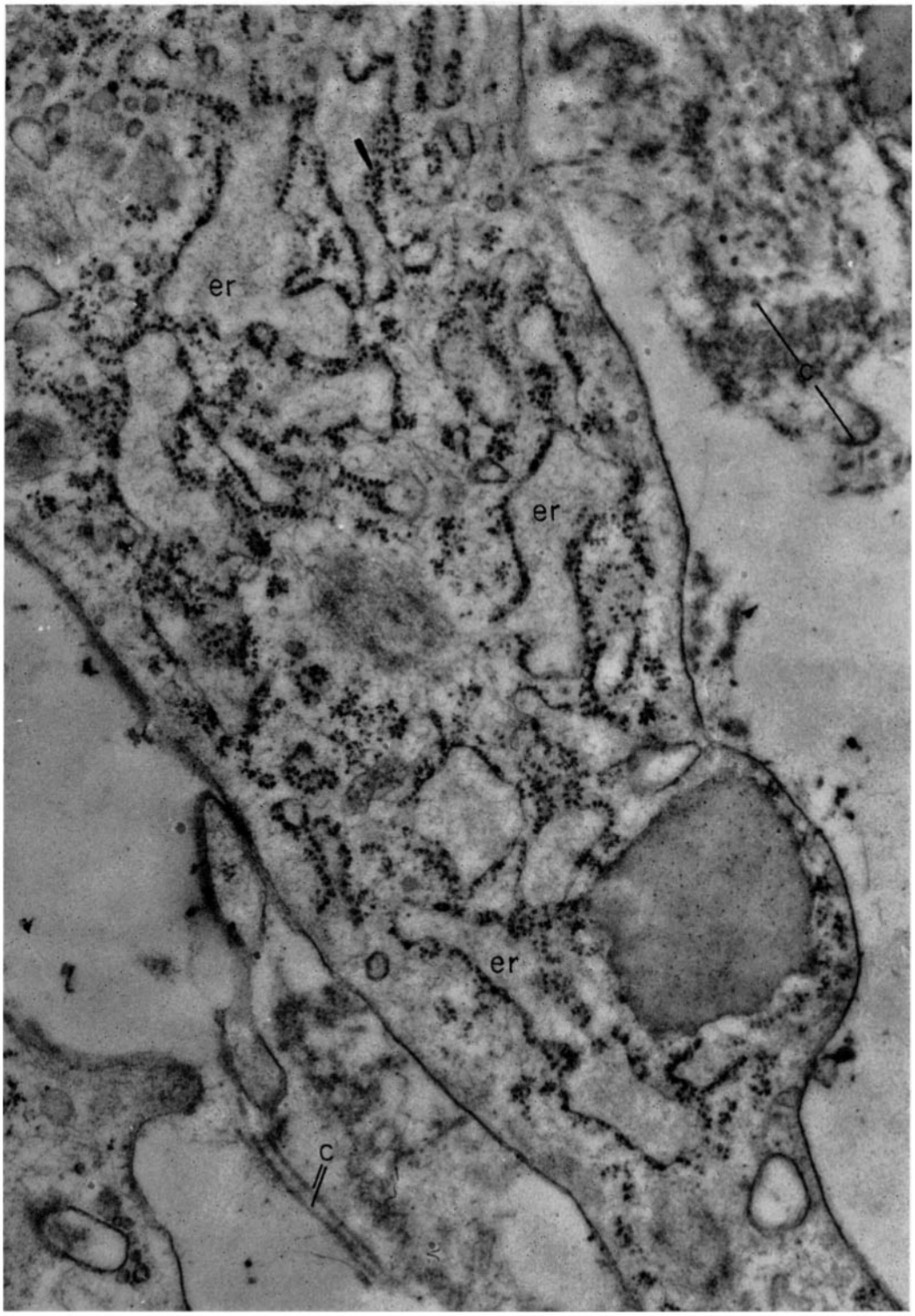


FIGURE 6 Another region from a 12-hour recovery wound displays parts of two cells. The profiles of endoplasmic reticulum (*er*) of the cell in the center appear to be more intercommunicated. Here again, collagen fibrils (*c*) cut both longitudinally and transversely can be seen in the extracellular regions, together with numerous fine filaments. × 33,000.

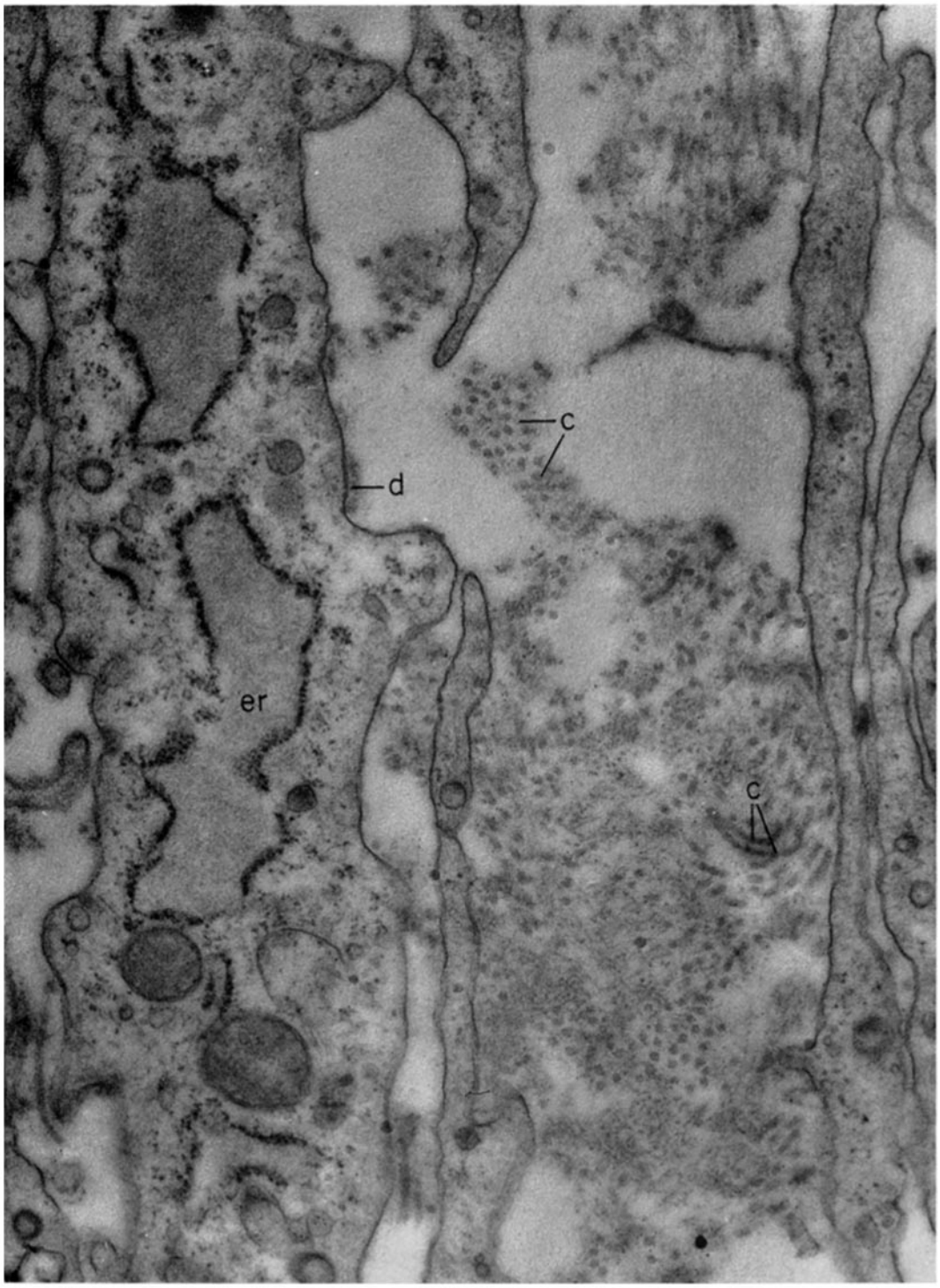


FIGURE 7 An electron micrograph of a wound 24 hours after ascorbic acid administration. At this time the endoplasmic reticulum (*er*) of the fibroblasts appears similar to that seen in cells of wounds from normal animals. Numerous mature collagen fibrils (*c*) are present extracellularly. Many smaller fibrils, possibly collagen, although not identifiable, are intermixed with the larger fibrils. Extracellular condensations (*d*), previously described in collagen-forming cells, adjacent to the plasma membrane are also visible. × 30,000.

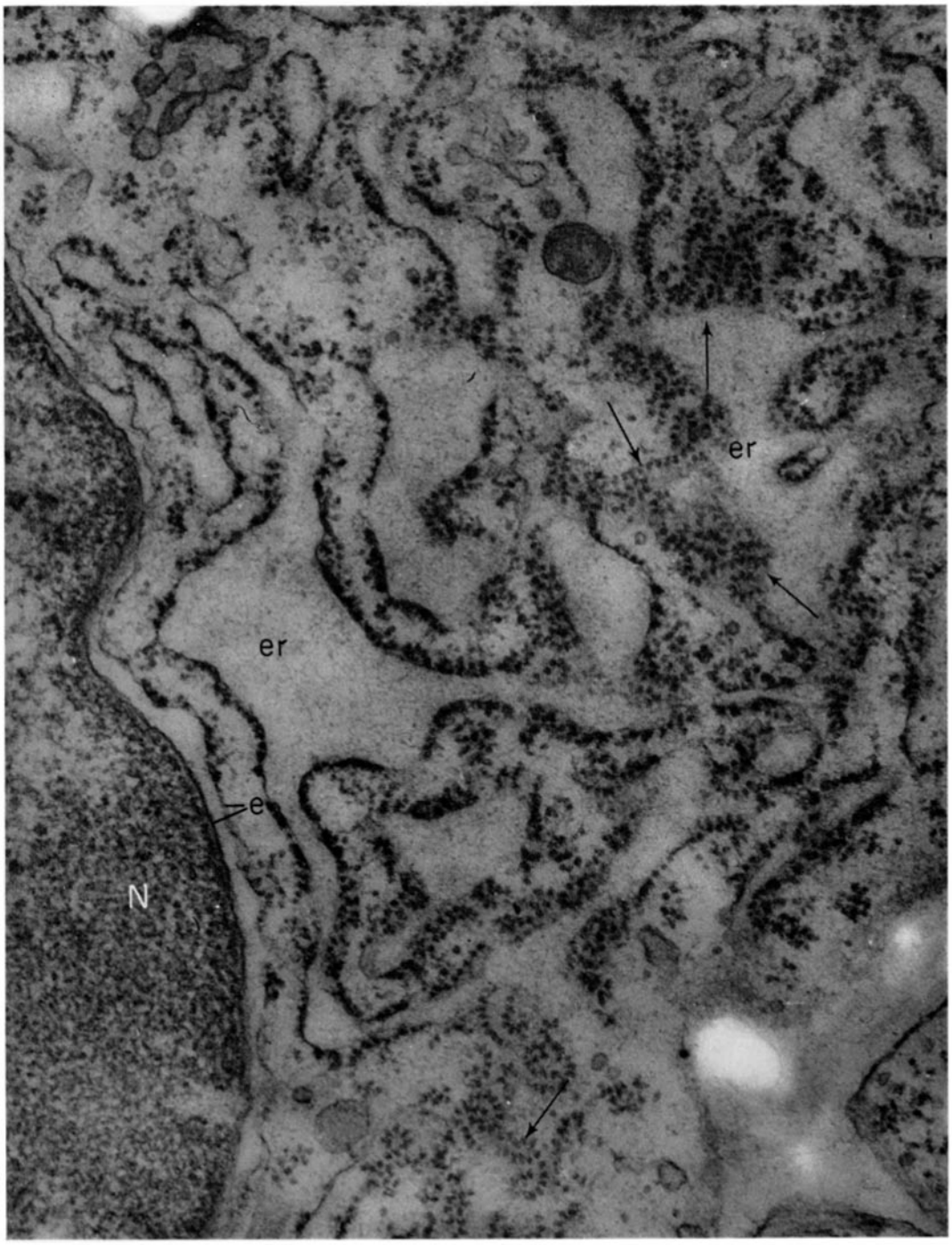


FIGURE 8 A micrograph from part of a fibroblast in a wound 24 hours after ascorbic acid administration. Part of the nucleus (*N*), nuclear envelope (*e*), and endoplasmic reticulum (*er*) are visible. The endoplasmic reticulum membranes have been cut tangentially in several regions and the ribosomal orientation on the membranes is observable. By this time, the typical paired rows are routinely present (arrows) and can be seen to take curved forms. × 54,000.

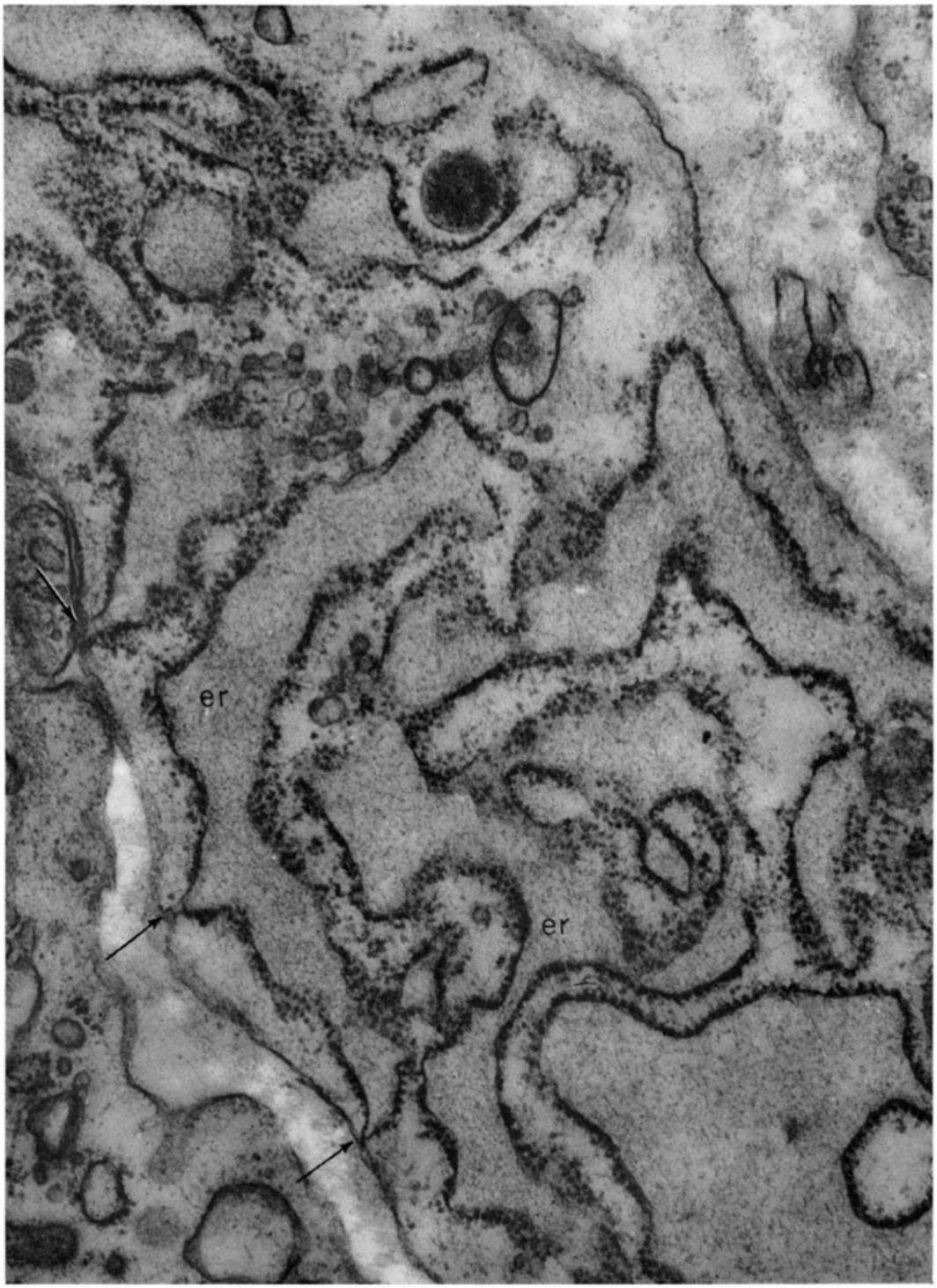


FIGURE 9 An electron micrograph of a cell from a 24-hour treated scorbutic wound. Three regions of approximation of endoplasmic reticulum (*er*) to the plasma membrane of this cell are present (arrows). In these zones, the ribosomes are fewer or are missing, and the membranes appear to merge in two of these regions. Stained with lead. × 48,000.

of the ribosomes of the ergastoplasm is lost in scorbutic fibroblasts. Furthermore, this configuration begins to reappear in a few areas very early (4 hours) after ascorbic acid administration. The return of this orientation increases with time, so that within 12 hours many of the cells show such an aggregation. Concomitant with the reorientation of the ribosomes, extracellular collagen fibrils begin to appear. This observation could either be coincidental or could reflect a direct relationship between these two events. Within 24 hours, very few fibroblasts exhibit the alterations seen in scurvy, insofar as the arrangement of the endoplasmic reticulum is concerned. That messenger RNA interacts with ribosomes and provides the assembly mechanism for protein synthesis is well established for bacteria. A similar mechanism is assumed to be operative in mammalian cells which synthesize cytoplasmic protein, such as the reticulocyte. If several ribosomes enter into such a complex, then their location and apparent pairing in rows on the ergastoplasmic membranes in normal fibroblasts, actively forming collagen, could reflect the presence of a usual messenger RNA. The restoration of this relationship after vitamin C is administered is presently compatible with one or another of the four following possibilities.

1. In scurvy there is a reduction in quantity or an alteration in quality of messenger RNA.
2. There is an alteration in the properties of the ribosomes *per se*.
3. There is an injury to the membranes of the coarse endoplasmic reticulum.
4. The lack of hydroxylation of proline and lysine in scurvy (15–19, 27–29) represent, in essence, an amino acid deficiency, and could be reflected in a disruption of the architecture of the ribosomal aggregates.

Any one of these could alter synthesis of the specific proteins produced by the fibroblast. It is not possible, presently, to adduce any direct evidence for propositions one, three, and four, but there is evidence for the second possibility: Smuckler and Benditt (52) recently found in toxic liver injury that the properties of the liver cell ribosomes were altered as shown by ultracentrifugation and their capacity to incorporate amino acids.

It is surprising that, so far, attention has been paid to unattached polysomes in contrast to those which are attached to the endoplasmic reticulum of cells synthesizing protein for export. Such investigations would undoubtedly yield further information on protein synthesis in these systems.

Configuration of Cisternae

In addition to the change in the pattern of the ribosomes on the cisternal membranes, the cisternae themselves demonstrate a marked alteration during scurvy. They are dilated and rounded and return to normal within 24 hours after giving vitamin C. This rounding is a feature shared with liver endoplasmic reticulum in CCl_4 poisoning associated with decreased protein synthesis (30). Such a cisternal alteration could possibly reflect a change in the components of the membrane itself, so that the ribosomal configuration is altered (as discussed above in scurvy), or so that ribosomes are detached from the membranes as in carbon tetrachloride intoxication. Unfortunately, no additional evidence relating to a possible membrane alteration is presently available.

The resumption of collagen synthesis by the fibroblasts is accompanied by the appearance of long, narrow, interconnected cisternae in these cells. In those regions in which the cisternal membranes approach or "point" to the plasma membrane, two observations are pertinent. The first is the appearance of a series of vesicles that seem to form a line between the ergastoplasm and the plasma membrane. The second, seen in Fig. 9, is the appearance, in many regions, of cisternal membranes approaching very close to the plasma membrane. In these zones, the membranes are characteristically void of their granules and have small out-pouchings which appear to merge in some areas with the plasma membrane. This is a *very common* and consistent finding in a large number of electron micrographs of the wounds 24 hours following ascorbic acid therapy, where it is probable that collagen synthesis is proceeding at a very rapid rate. These regions may represent sites of release of synthesized peptides or proteins into the extracellular spaces.

The only other site in the fibroblasts in which the cisternal membranes of the endoplasmic reticulum are void of ribosomes is the area where the ergastoplasm merges with the Golgi zone (Fig. 2 *a*).

Lipid

The lipid deposits in the majority of scorbutic fibroblasts begin to disappear within 24 hours

following ascorbic acid administration. By 48 hours, only an occasional lipid droplet is visible within the cells. We have mentioned elsewhere for the fibroblast (8), and the liver (43), that this may represent a depression of lipid transport as lipoprotein, with decrease in protein synthesis and transport.

Extracellular Changes

The nature of the extracellular filamentous material in scorbutic wounds has been a subject of interest for many years. Wolbach (5, 6) thought that it represented a gelatinous precursor of collagen and put forth his well known "gelation hypothesis" on the basis of its staining and the relatively rapid appearance of collagen following ascorbic acid administration.

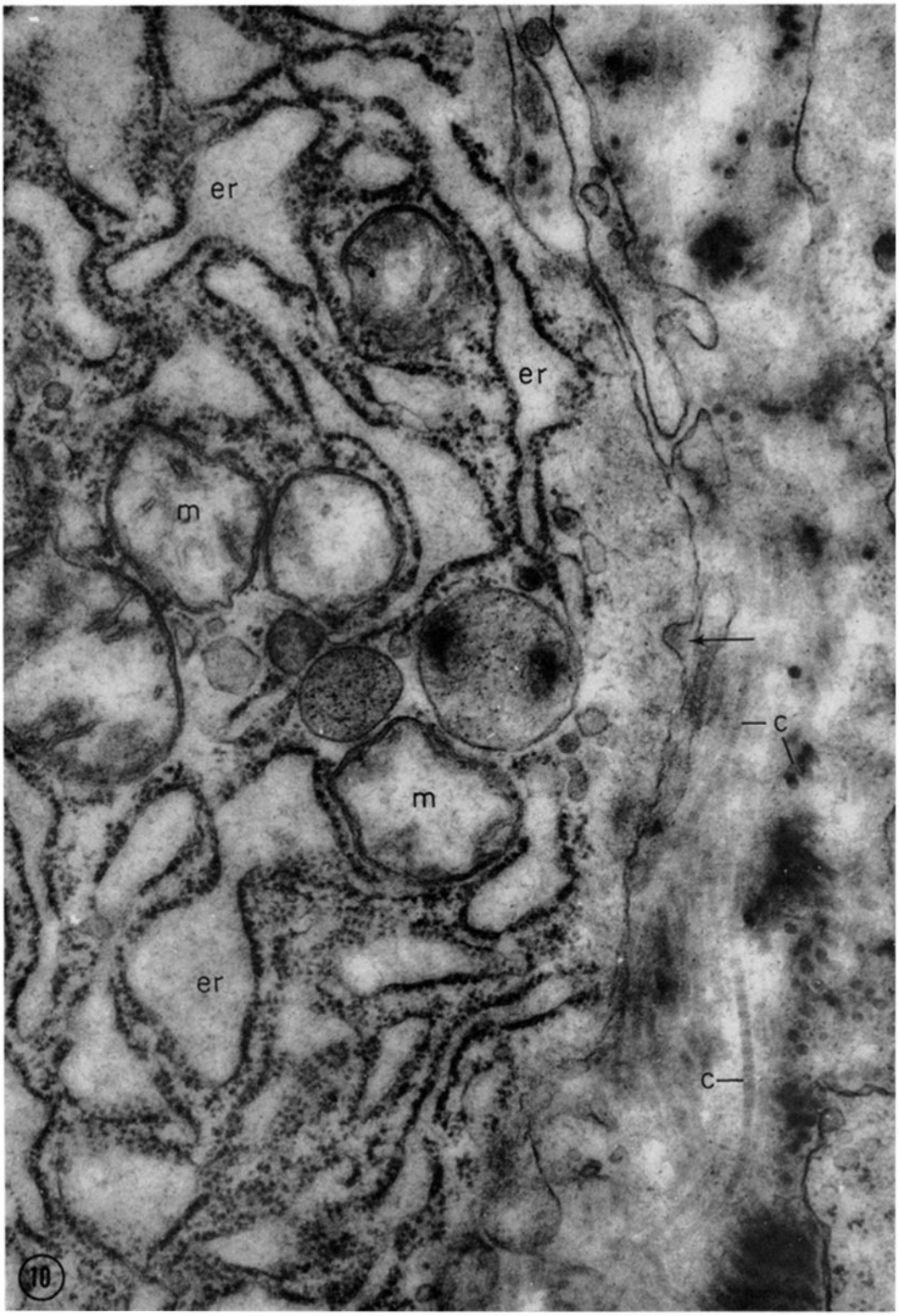
The histochemical and enzyme digestion studies presented above demonstrate that this material contains little tryptophan and that it is susceptible to digestion by trypsin, but not by collagenase. Fibrin present in the wounds stained deeply for tryptophan (indole) with the Adams' method (31) as well as with the fibrin stains, and acted as a control for the staining of the scorbutic material. In addition, fibrin could be identified in the electron micrographs by its characteristic banding. Using the histochemical technique, the scorbutic material may appear to contain a very small amount of tryptophan as indicated by a faint blue haze over all of the material in the extracellular spaces. On the other hand, this could well be due to the diffuse presence of small amounts of fibrinogen or other plasma proteins.

The autoradiographic studies utilized tritiated proline known to be the precursor of both collagen proline and hydroxyproline (15, 16). The results of these experiments can be interpreted in at least 3 possible ways.

1. The apparent retention of the label by the extracellular material, following ascorbic acid administration, associated with the appearance within these regions of collagen fibrils, could be interpreted as conversion of this material to collagen (37). However, further observations in our laboratory, as well as those of others (28, 35), do not support this concept.
2. A second possible interpretation is related to the fact that these wounds undoubtedly contain serum proteins, attributable to the increased permeability occurring in scurvy. Proline is present in considerable quantity in plasma proteins. The slowly increasing amount of label in the extracellular material, associated with an apparently stable amount of cellular label, could be interpreted as leakage of these proteins into the wound. Although a turnover of label could occur, it would not be detectable by this technique.
3. A third possibility is that the scorbutic cells may be synthesizing an unusual protein. Scorbutic fibroblasts incorporate proline and release it into the extracellular filamentous material. Electron microscope autoradiographic observations (51) indicate that the label passes through the endoplasmic reticulum and appears over the extracellular material at a somewhat slower rate than that observed in normal animals (9). If this kind of evidence can be interpreted as indicative of protein synthesis, then this could represent either a protein other than collagen, normally produced by fibroblasts, or it could be an abnormal protein.

How the scorbutic filamentous material disappears in the short space of 24 hours is difficult to explain. There is no evidence of phagocytosis of this material, although erythrophagocytosis and ferritin-containing vacuoles are present in macrophages in the scorbutic wounds. No new phagocytic cells are observable in the recovery wounds to explain this phenomenon. Yet the quantity of this material appears to decrease with

FIGURE 10 Part of a wound 72 hours following vitamin C administration. The fibroblast is indistinguishable from those seen in non-deficient animals. Extensive, long, interconnected profiles of endoplasmic reticulum (*er*) are present, as well as vesicles, mitochondria (*m*), and a small caveola (arrow). Collagen fibrils (*c*) with their characteristic periodicity are cut both transversely and longitudinally and appear to fill the extracellular regions. × 52,000.


er
er
m
m
er
c
c
10

increase in collagen. The possibility that the fibroblasts are removing it and perhaps re-utilizing this material in collagen synthesis cannot be excluded.

General Considerations

Two major hypotheses for the extremely rapid appearance of collagen observed following the administration of vitamin C have been advanced. They are:

1. A collagen precursor is synthesized in scurvy; this precursor contains proline that is peptide-bound, approximately half of which becomes hydroxylated upon administration of ascorbic acid (16, 17, 24, 26, 27).
2. Proline and hydroxyproline enter the collagen molecule separately; both are derived from the proline pool which is available for this purpose (13, 14, 28). Ascorbic acid is probably involved in both the hydroxylation of proline to hydroxyproline, and lysine to hydroxylysine, and the subsequent incorporation of these amino acids into the collagen molecule (14, 20, 29).

The possibility of the formation in scurvy of a precursor of collagen, rich in proline and poor in hydroxyproline, was explored by Gould and Woessner (17). They reported observations of extracts from healing wounds in scorbutic guinea pigs which demonstrated an increase in hydroxyproline with a concomitant decrease in proline and glycine, following ascorbic acid administration. This appeared consistent with their hypothesis. However, the amino acid composition of protein extracted by methods presumed to extract collagen was not consistent with such a collagen precursor (27). Gross failed to observe in neutral salt extracts of scorbutic skin any proline-rich material (35). Later attempts by Gould *et al.* (18, 19) were also unsuccessful in recovering a protein rich in proline and glycine. Chen and Postlethwait (23) have intimated that they have data which suggest the presence of such a substance but, to date, have not presented them in full.

Robertson, Hewitt, and Herman (28) were unable to find a proline-rich, hydroxyproline-poor precursor in scorbutic carrageenin granulomas following administration of C^{14}-proline concurrently with ascorbic acid to scorbutic animals. The specific activity of hydroxyproline in the granulomas was higher than that of the proline. Had an accumulation of a proline-rich precursor taken place, the specific activity of hydroxyproline following administration of vitamin C would have been expected to be low. Mitoma and Smith (29), using the same experimental situation, found a decreased specific activity for both proline and hydroxyproline in scurvy, and could find no indication of a defect in hydroxylation of proline as determined by measurements of urinary hydroxyproline. In contrast to this, however, are the findings of Martin, Mergenhagen, and Prockop (36) who noted a significant decrease in urinary hydroxyproline in scorbutic guinea pigs. Stone and Meister (14) have studied this problem by measuring the amount of tritiated water formed from tritiated proline in granulomas from normal, from deficient, and from ascorbic acid–treated animals. Additional evidence relating the cellular alterations in scurvy to the absence of hydroxyproline and hydroxylysine comes from the studies of Manner *et al.* (32–34). They have been able to identify the presence of *s*-RNA-hydroxyproline and *s*-RNA-hydroxylysine. In contrast, Peterkofsky and Udenfriend present evidence in a cell-free system, derived from chick embryos, that hydroxylation occurs in small peptides in the microsomes (57). Their findings would indicate that hydroxylation occurs at a later stage than that indicated by Manner *et al.* (32–34). Although the evidence appears conflicting, the presently available findings are distinctly in favor of the following concept: Proline is hydroxylated prior to incorporation into protein. Both events occur within the cell.

The present observations show an alteration, in scorbutic fibroblasts, in the structural elements of the cell involved in protein synthesis, specifically in the arrangement of the ribosomes in relation to the membranes of the endoplasmic reticulum. The normal pattern is restored after vitamin C administration. Both this morphological evidence and the biochemical findings cited above place the lesion in scurvy within the fibroblast. It remains to be seen whether or not the alterations in cell structure are directly related to these alterations in chemical functions. Our observations demonstrate that, subsequent to giving ascorbic acid, restitution of the normal configuration of the cellular apparatus for protein synthesis occurs concomitant with the appearance of collagen. If this is not simply coincidence, it would seem likely

that the early formation of collagen is due to its very rapid synthesis, and not to the conversion of an incomplete extracellular precursor.

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