WOUND HEALING AND COLLAGEN FORMATION

III. A Quantitative Radioautographic Study of the Utilization of Proline-H³ in Wounds from Normal and Scorbutic Guinea Pigs

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ABSTRACT

The sequence of incorporation and utilization of tritium-labeled proline has been examined in healing wounds from normal and scorbutic guinea pigs. Linear incisions in the skin of the animals were allowed to heal for 7 days. Each animal was given proline-H³, and the wounds were excised 30 minutes, 1 and 4 hours, 1, 3 and 7 days after proline administration. The tissues were fixed in osmium tetroxide, fixed again in neutral buffered formalin, embedded in epoxy resin, and sectioned at 1 micron thickness. The sections were coated with nuclear track emulsion, exposed, developed, and stained. The results of grain counts were quantitated as the number of counts per unit area overlying cells, fibers, etc. In both groups the proline reaches a maximum over the fibroblasts within 4 hours and subsequently disappears from the cells. Concomitantly, the proline reaches a maximum over the collagen (in normal animals) and extracellular fibrillar material (in scorbutic animals) by 4 hours, where it remains. The modified technique of radioautography used in this study allows not only resolution of approximately 1 micron, but also minimal background, decreased artifact, and a clear separation of the randomly situated elements within the wounds so that grain counting is facilitated. The results correlated with previous electron microscopic studies are consistent with the utilization of proline by the fibroblasts and its incorporation into collagen (in normal animals) and into the extracellular, fibrillar, non-collagenous material seen in scorbutic animals.

INTRODUCTION

Observations of the fine structure of fibroblasts in wounds removed from normal and ascorbic acid-deficient guinea pigs demonstrate that these cells contain an abundant rough surfaced endoplasmic reticulum (1, 2). This system of elements has been related to the synthesis of protein and is identical with the microsomes of cell fractionation (3, 4, 5). Keller, Zamecnik, and Loftfield (6) indicated the microsomal fraction to be the site of active incorporation of amino acids into protein.

The radioautographic studies of Carneiro and Leblond (7) utilize this previously mentioned knowledge. These investigators studied the incorporation of tritium-labeled glycine into the dentin of the developing incisor, and into developing bone of the mouse. The organic matrices of both bone and dentin are largely collagen. In both sites they found that the amino acid was taken up by the odontoblasts and osteoblasts 30 minutes after administration, and subsequently was found in the dentin and bone, respectively, within 4 hours.

It is the purpose of these studies to establish the sequence of utilization of tritium-labeled proline in healing wounds. The examination of this phenomenon in both scorbutic animals and animals fed a normal diet permits the comparison of a control situation with a situation in which collagen synthesis can be specifically abolished. In the present study, the phenomenon is examined with a modified radioautographic technique which provides not only resolution of ca. 1 micron, but also decreased artifact, as well as a clear separation of cells from each other and from the extracellular materials formed during the process of fibroplasia.

MATERIALS AND METHODS

Animal Preparation and Wounding

Wound healing was observed in two groups of female guinea pigs (four animals per group) weighing 250 to 300 grams each. All of the animals received initially the same diet (Nutritional Biochemicals), containing 20 mg of ascorbic acid per 20 gm of diet, to accustom them to the consistency of the food. After 5 days the second group was switched to an identical diet, save for a complete absence of ascorbic acid. After the animals had been on their respective diets for 8 days, the hair was removed from the dorsal skin and 8 linear incisions approximately 1 cm long were made. The wounds were allowed to heal by first intention for 7 days. At this time each animal was given L-proline-3,4-H³, intraperitoneally (New England Nuclear Corp.) (either 8 μc/gm body weight, or 12 μc/gm body weight). The tissue was removed by taking an ellipse of skin around each wound at 30 min., 1 and 4 hours, 1, 3, and 7 days after administration of the proline-H³. The wounds were then cut transversely with sharp razor blades into small cubes approximately 2 mm in dimension, and were fixed and processed as previously stated for electron microscopy (1). Sections approximately 1 micron thick were cut on

the Porter-Blum microtome with glass knives. Individual sections were floated out on clean glass microscope slides. Excess water was removed and the slides were dried on a hot plate to firmly fix the section to the slide. Upon completion of all of the sectioning, each slide was coated with nuclear track emulsion (Eastman, NTB-3). The emulsion was heated to 40°C, a drop of emulsion was placed on one end of the microscope slide, and, utilizing surface tension, the emulsion was spread with a glass rod as uniformly as possible over the entire slide, including the tissue section. The slides were air dried and stored in a light-tight box containing a small amount of Drierite. After exposure for periods of 6, 8, and 10 weeks (for animals receiving 8 μc/gm body weight), and 4, 5, and 6 weeks (for animals receiving 12 μc/gm body weight), the slides were developed in Dektol for 2 minutes and fixed for 2 minutes. They were then washed for 30 minutes and air dried. After drying, each slide was individually stained with azure II-methylene blue (14).

The numbers of grains overlying cells, collagen, and extracellular material were then counted in the following manner: Random photomicrographs were taken of the wound areas using the Zeiss photoscope at a magnification of 400. Sections from two blocks of each wound were examined and photographed. Five random photomicrographs were taken of each wound, and all pictures were enlarged five times. Similar pictures were taken of five areas immediately adjacent to the sections for background counting. The pictures were divided into quadrants to facilitate grain counting, and the numbers of grains were counted over nuclei and cytoplasm, extracellular space, collagen, (extracellular material in scorbutic wounds), erythrocytes, capillaries, and all other elements. A clear plastic sheet containing a grid with 180 intersections was placed over each picture, and the relative area occupied by cells, fibers, and extracellular space was determined. From this the absolute numbers of grains over cells/unit area, fibers/unit area, etc. were determined. These counts were then corrected for background by subtracting the mean counts of the background for each section from the total numbers of counts determined for the different

FIGURE 1

An electron micrograph of a 7-day wound from a normal animal. Two fibroblasts (F) with their extensive endoplasmic reticulum (er), mitochondria (m), and peripheral intracytoplasmic aggregates of filaments (f) are contrasted with a second cell type which lacks these characteristics and contains a much denser cytoplasmic matrix. The Golgi complex (G) and mitochondria (m) of this cell are also apparent. Centrioles (ce) are seen in this cell as well as in one of the fibroblasts. A membrane-bounded space (arrow) containing collagen fibrils is interpreted to be an invagination of extracellular space into this cell. The extracellular spaces contain large numbers of collagen fibrils (c) cut both transversely and longitudinally. × 20,000.
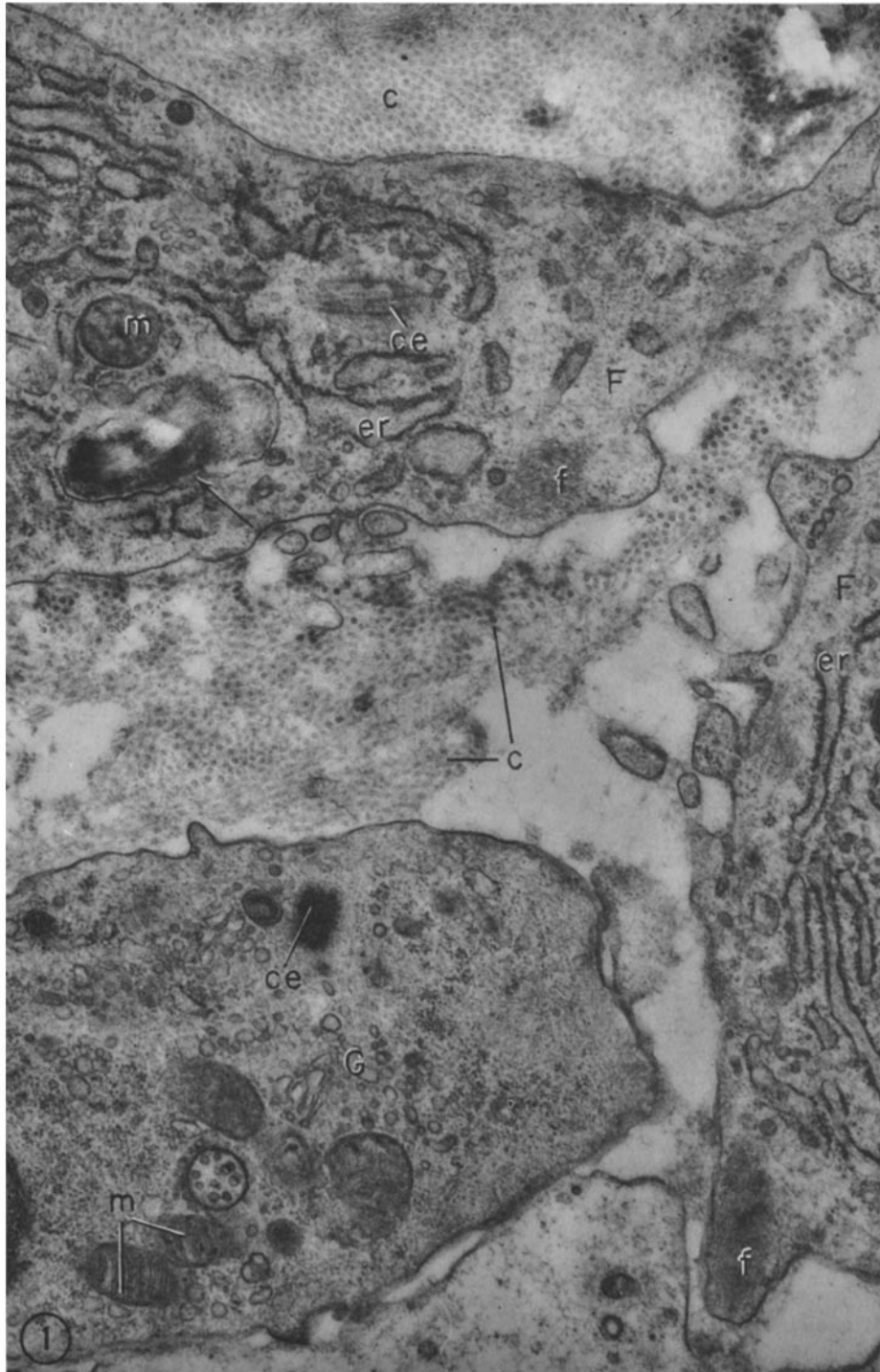
elements. Hence, at every time interval the corrected counts per unit area occupied by cells, fibers, etc. were determined in each case.

Criteria for Determination of the Scorbutic State of the Animals on the Scorbutigenic Diet

The epiphyses of the long bones and the odontoblasts of the teeth of each animal were examined as in the previous work (2) to ascertain that the animals

Counts per unit area

Mean No. of counts per unit area in wounds following administration of proline-H³ to a group of normal animals

Mean No. of counts per unit area in wounds following administration of proline-H³ to a group of scorbutic animals

o— Cell
△—— Collagen

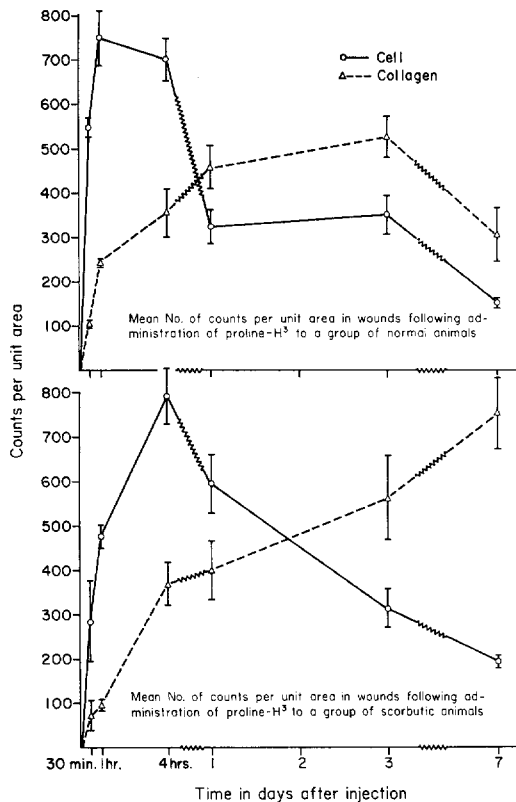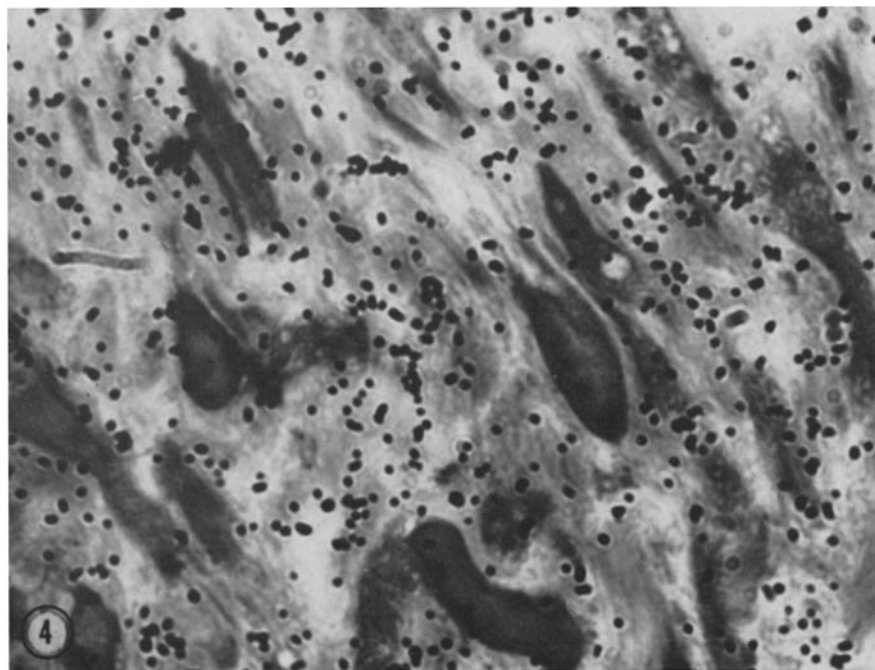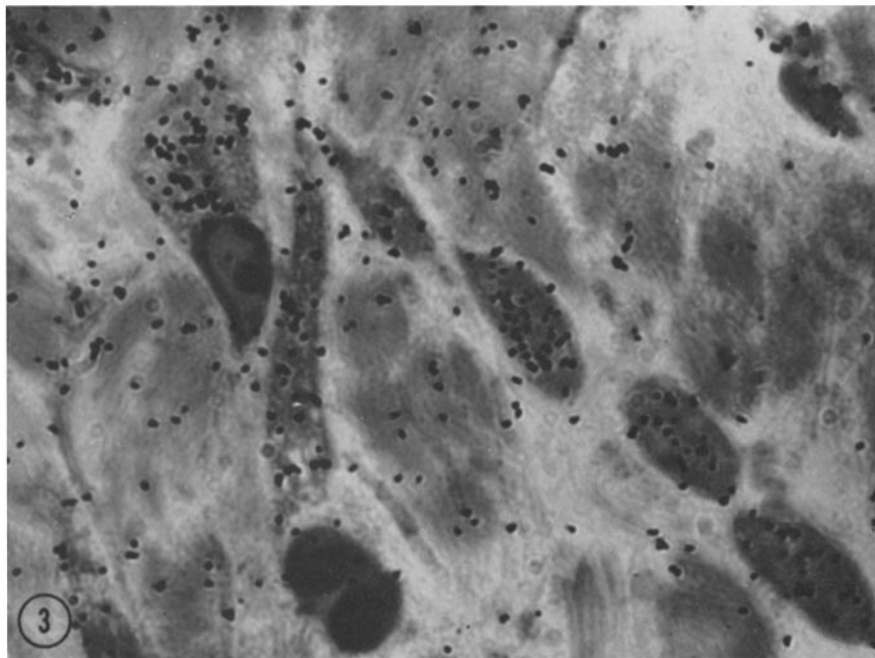30 min. 1 hr.   4 hrs.   1   2   3   7

Time in days after injection

FIGURE 2

These graphs are a composite of the numbers of counts obtained from the normal and scorbutic animals, respectively, after 5 weeks' exposure of the slides. The points represent means, and the bars standard errors of the mean. The abscissa represents the time periods after intraperitoneal injection. The major differences noted between the normal and scorbutic animals are (a) a decreased rate of uptake and release of the label by the scorbutic fibroblasts as related to the controls; (b) a decreased rate of uptake by the scorbutic fibrillar material as compared to the collagen during the early time periods; and (c) a decrease in the amount of label in the collagen contrasted with an increase in the scorbutic fibrillar material from the 3rd to the 7th day.

receiving the ascorbic acid–deficient diet were truly scorbutic.

OBSERVATIONS

Animals on Normal Diet

Electron micrographs (Fig. 1) typical of the cell types seen in wounds from normal animals demonstrate the extensive rough sufaced endoplasmic reticulum of the fibroblasts and the peripheral filamentous aggregates previously described, as well as the numerous extracellular collagen fibrils seen in 7-day-old wounds (1).

The results of the grain counts from the control wounds following the administration of proline-H³ are recorded in the graph in Fig. 2. The appearance of these wounds is as follows:

At 30 minutes the majority of the fibroblasts display grains located over their cytoplasm. A few grains are present over the collagen and extracellular space. By 1 hour, the number of grains over the cells is markedly increased concomitant with an increase over the collagen fibers (Fig. 3). By 4 hours, the number of grains over the cells has reached a maximum, as has the grain density over the collagen fibers as indicated by the means which are not significantly different from each other as shown by the bars which represent the standard error of the mean. By 24 hours, the grain density is markedly decreased over the cells while it remains at a high level over the collagen fibers. From the 1st day on, the number of exposed grains remains relatively stable over both cells and collagen. By the 7th day after proline-H³ administration, the location of grains over cells and collagen appears similar to that at the 24-hour period (Fig. 4), with some decrease in the total counts over both.

Thus, proline appears to enter into the fibroblasts by 30 minutes and reaches a maximum by 4 hours. However, by 1 hour the radioactivity has already appeared in the extracellular material. By 24 hours, the bulk of the radioactivity is present in the extracellular material, where it remains.

Animals on the Scorbutigenic Diet

An electron micrograph (Fig. 5) of a 7-day-old wound from a scorbutic guinea pig demonstrates the "rounding up" of the endoplasmic reticulum of the fibroblasts and the presence of large amounts of filamentous material, not identifiable as collagen, in the extracellular spaces.

FIGURE 3

This light micrograph displays a representative region of a control wound 1 hour after the administration of proline-H³. The majority of silver grains are present over the fibroblasts. The somewhat fuzzy appearance is due to the fact that neither the grains nor the cells are in true focus. × 2000.

FIGURE 4

A light micrograph from a representative region of a control wound 7 days after proline-H³ administration. The majority of silver grains are present over the collagen fibers rather than the cells, in contrast to the 1 hour time period. × 2000.
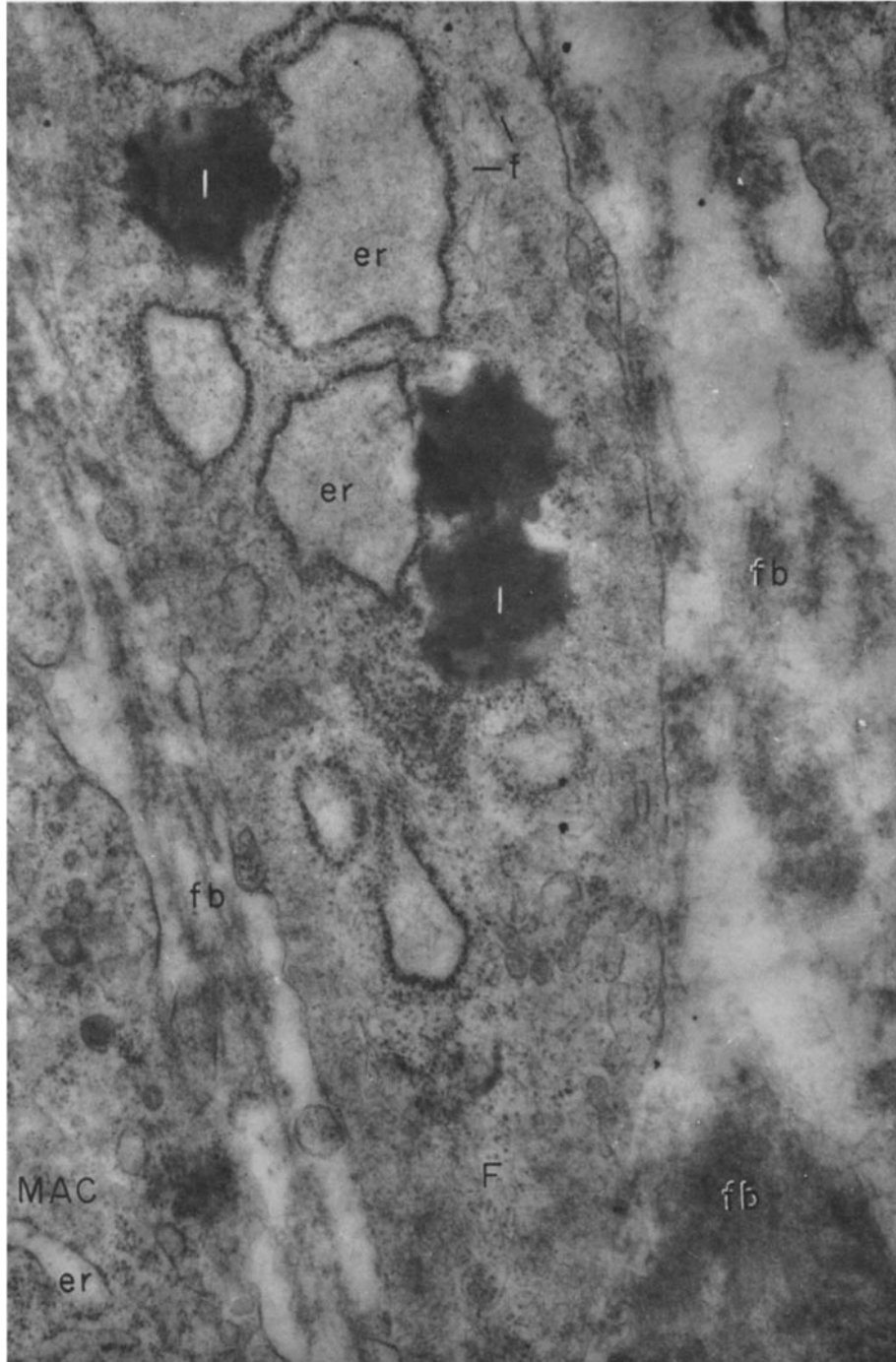
This electron micrograph is from part of a 14-day scorbutic wound. A fibroblast (F) is juxtaposed against a macrophage (MAC). The fibroblast is characterized by its rounded cisternal profiles (er), lipid deposits (l), and intracytoplasmic filaments (f). Cisternae of endoplasmic reticulum (er) and small vesicles are seen in the macrophage. The extracellular spaces contain the non-banded fibrillar material (fb) so characteristic of the scorbutic wounds from the 3rd day on. × 32,000.
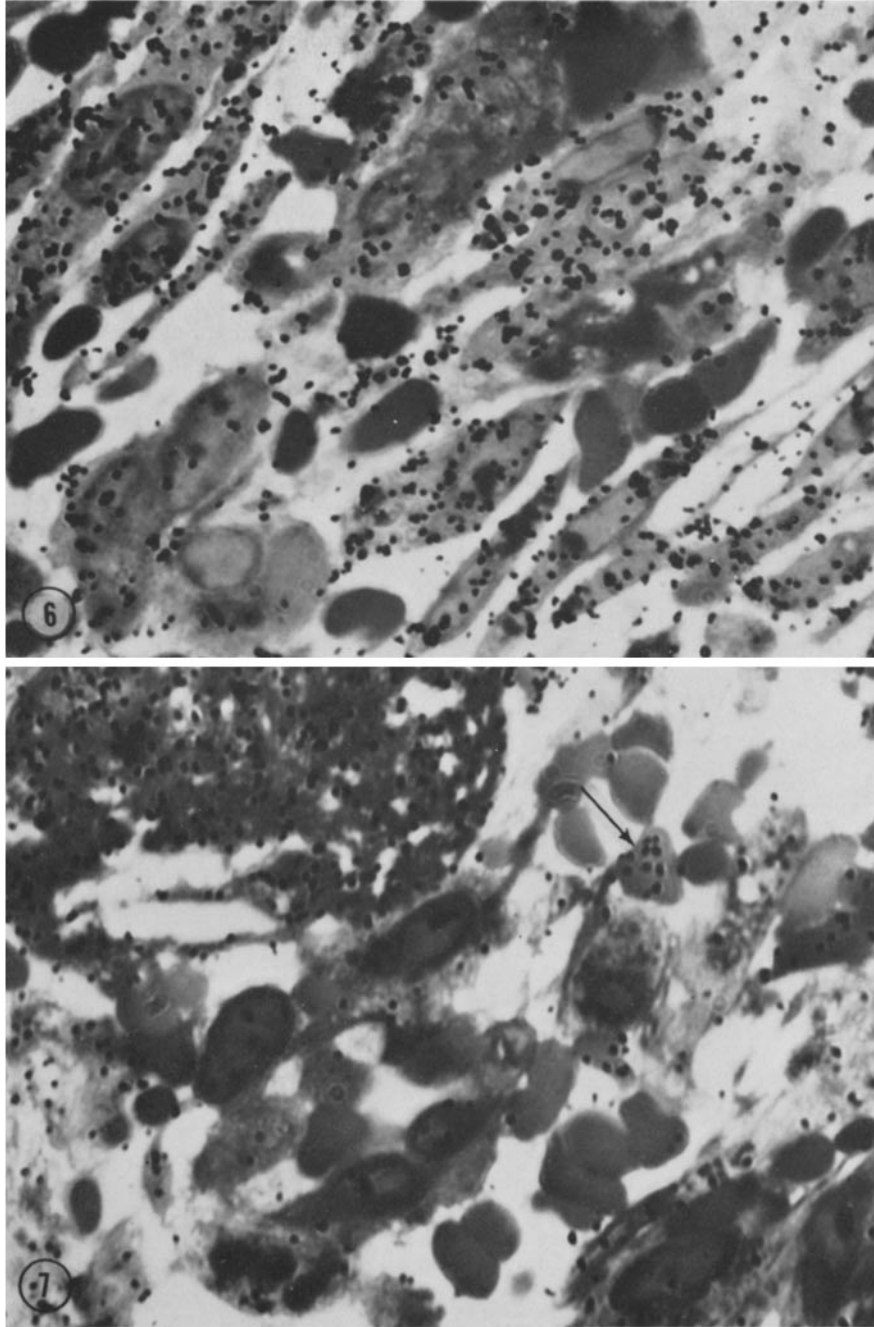
FIGURE 6

A light micrograph from a representative region of a scorbutic wound 4 hours after proline-H^3 administration. The majority of the silver grains are present over the fibroblasts. × 2000.

FIGURE 7

A light micrograph from a representative region of a scorbutic wound 7 days after proline-H^3 administration. The majority of silver grains are present over the extracellular fibrillar material. A few erythrocytes (arrow) can be seen to be labeled as well. × 2000.

The sequence of events in the scorbutic animals is similar to that seen in the controls, but the rates of uptake and release of proline-H^3 are slowed. The results of the grain counts from this group are represented in Fig. 2.

At 30 minutes and 1 hour the majority of the fibroblasts display grains overlying their cytoplasm. A few grains are present over the extracellular material. By 4 hours the grain density over the cells increases to a maximum approximating that seen after 4 hours in the control animals (Fig. 6). At 24 hours the proline concentration decreases in the cells and is increased in the extracellular dense material previously described. The cells in the wounds at this stage appear to contain more label than those in the 24-hour wounds from the control animals. After 24 hours the grain density decreases over the cells and slowly increases to stabilize eventually over the extracellular material. By the 3rd day the number of counts over the cells is relatively low while it is intense in many regions over the extracellular material and appears to increase slowly to the 7th day (Fig. 7).

Hence, in scorbutic animals the processes of entry of proline into the cells and exit from them to the fibrillar, extracellular material differ from those in control animals only in the delay in uptake and slower release of this amino acid by the cells. It is significant that the label is apparently bound in some fashion within the extracellular material. In both groups of animals, the mean numbers of counts per unit area of cells and fibers were of a similar order of magnitude at most of the time periods examined except during the period from 3 to 7 days.

DISCUSSION

The observed apparent march of the proline through the cells and into the extracellular material coupled with the electron microscope observations are consistent with the following conclusions: (a) In guinea pigs fed a normal diet, the fibroblast synthesizes the collagen unit and secretes it into the extracellular space where fibril aggregation takes place. (b) In scurvy, the fibroblast also utilizes proline in the synthesis of a material which appears as a filamentous non-banded substance in the extracellular space.

Proline and hydroxyproline together constitute approximately 25 per cent of the collagen molecule. In 1944, Stetten and Schoenheimer (8) showed that upon administration of proline-N^{15}

to rats a significant amount of hydroxyproline in forming collagen was labeled within 3 days. Stetten (9) found later that hydroxyproline-N^{15} was not utilized in the formation of collagen in these animals. From these experiments, she concluded that collagen hydroxyproline was derived from proline destined to be both proline and hydroxyproline. Smith and Fitton-Jackson (10) subsequently supported Stetten's conclusions when they found that chick osteoblasts in tissue culture were able to convert proline-C^{14} to hydroxyproline-C^{14}. Administration of large amounts of unlabeled hydroxyproline did not affect the conversion and incorporation of the C^{14} into hydroxyproline of the collagen formed by these explants. With the knowledge that the fibroblast contains the morphologic apparatus associated with protein synthesis, Lowther, Green, and Chapman (11) and Eastoe (12) performed cell homogenization and fractionation with a biochemical assay of the resultant fractions. The former investigators also examined the uptake of proline-C^{14} by these fractions and found a significantly higher specific activity of labeled hydroxyproline in the microsomal fraction. Peterkofsky and Udenfriend (13) demonstrated that fortified microsomes in a cell-free system from chick embryo homogenates were able to convert proline to hydroxyproline, which was found in microsomal protein. Since proline serves as the source for both collagen proline and hydroxyproline, it is an excellent agent with which to study collagen synthesis radioautographically.

The radioautographic observations described here show no difference in the amount of label taken up by the fibroblasts of scorbutic wounds when compared to the fibroblasts in the control wounds. However, a difference in the rate of uptake and release of the label by the scorbutic cells was observed in our studies. The rough surfaced endoplasmic reticulum is extensively developed in both groups of cells although altered morphologically in scurvy. In the scorbutic fibroblasts this system of elements appears in the form of rounded vesicular structures rather than as a continuous system of channels (2). The relation of this system to protein synthesis (3–6, 11, 12), the lack of intracytoplasmic collagen fibrils, and the passage of the isotope are consistent with the synthesis, release, and extracellular aggregation of the precursors into fibrils in both cases.

It is not clear whether, after a period of time,

the label represents exclusively proline or hydroxy-proline and glutamic acid as well (which can be derived from proline). This information is necessary in order to understand the relationship of the hydroxylation of proline to collagen fibril aggregation. As yet we have no definitive information on this matter. There is controversy as to whether hydroxylation of proline occurs in scurvy where collagen formation can be specifically abolished (15–18). Further evidence as to the validity of the above conclusions will require separation, identification, and analysis of the fibrillar material present in the scorbutic wounds. At the moment, it seems unlikely that the proline-H^3 would be adsorbed merely to the collagen, or to the scorbutic fibrillar material, as we would expect any adsorbed or loosely bound material to be lost during the processes of fixation, dehydration, and embedding.

Finally, mention should be made of the techniques used for radioautography in this work. The use of epoxy resins allows thin sections which are stable and contain no tears or gross defects. Such sections permit clear morphologic separation of the various elements in a crowded milieu such as the healing wound. With a thin layer of emulsion the tissue and the silver grains are visible within the same focal plane. In addition, this technique provides tissue already prepared for further radioautography at the electron microscope level.

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