CONTROLLED FORMATION OF COLLAGEN AND RETICULUM. A STUDY OF THE SOURCE OF INTERCELLULAR SUBSTANCE IN RECOVERY FROM EXPERIMENTAL SCORBUTUS *

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Previous studies [1] showed that in the state of complete or absolute scorbutus formation of intercellular materials cannot take place, and that following the administration of antiscorbutics easily demonstrable amounts of intercellular substance are formed within 24 hours. This rapid formation of intercellular substance occurs in regions where mesenchymal cells have accumulated in continuation of normal growth activities, in repair of spontaneous lesions in consequence of scorbutus and in the organization of blood clots after excision of tissue. These facts have made it possible to obtain and to recognize, in preparations from regions of growth and repair, intercellular substances deposited at will through the administration of antiscorbutics. Furthermore, we are enabled to locate exactly where collagen forms in relation to cells and fibrin and to follow the sequences in morphology of intercellular substances to the final or mature state.

Inasmuch as there are several conflicting beliefs concerning the source of collagen and its relation to cells this communication is restricted to observations that are of value as premises for the solution of this problem.

Although a large amount of material from many guinea pigs was on hand, covering all stages in the repair of spontaneous lesions in scorbutus, a new series of guinea pigs was prepared in order to take advantage of the isolation of connective tissue cells which occurs in the avascular organization of blood clots in animals in absolute scorbutus. Absolute scorbutus we define as the stage in experimental scorbutus in which formation of intercellular materials either in growth or repair has ceased. Proliferative reparative cellular responses, epithelial and mesenchymal, are active. It is always accompanied by marked osteoporosis.

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The diet was that previously employed by Wolbach and Howe.[1] Guinea pigs of from 315 to 400 gm. initial weight were used. On the 23rd day of the vitamin C free diet a large piece was removed from the extensor thigh muscles of each animal, under aseptic precautions, and the skin incision sutured. The ensuing blood clot was allowed to undergo organization for periods of from 5 to 8 days before administration of orange juice. The duration of treatment with orange juice, before sacrificing the animal, varied from 24 to 96 hours. Control guinea pigs, *i. e.*, those that received no orange juice following the removal of muscle, were killed at the end of 7 to 10 days. From each animal, immediately after death, the experimental wound was excised, with the minimum amounts of surrounding normal tissues, so as to ensure the best possible fixation. The costochondral junctions were also saved and sectioned. Zenker's fixative was used for both types of material. Twenty-four hours fixation was sufficient to decalcify the osteoporotic ribs. All tissues were embedded in paraffin. The stains employed were a modified Giemsa stain [2] for general study, Mallory's phosphomolybdic acid anilin blue- acid fuchsin- orange G connective tissue stain,[3] Mallory's phosphotungstic acid hematoxylin,[3] and Foot's modification of the Bielschowsky-Maresch silver impregnation method [4] followed by Van Gieson's acid fuchsin-picric acid stain, or by Mallory's connective tissue stain.

The repair of the blood clot in absolute scorbutus begins promptly by the migration of fibroblasts from adjacent tissues into the clot, and the continued division of these cells. The fibroblasts are unaccompanied by collagen formation and they retain shapes resembling those of embryonic connective tissue cells, or fibroblasts in tissue plasma cultures (Fig. 1). Capillaries do not penetrate the clot for any considerable distance and are apparently unable to form, although closed columns of endothelial cells accompanied by fibroblasts do form. Islands of erythroblastic cells appear in the peripheral zone of the clot and in the adjacent tissue about capillaries, and apparently are derived from vascular endothelial cells whose initial reparative response to the experimental wound is multiplication. The fibroblasts apply themselves closely to fibrin when the latter is present. They probably follow fibrin strands in their movements (Fig. 2). The behavior of the cells invading the blood clot and the

microscopic picture they produce are similar to those arising in tissue plasma cultures. Fibroglia fibrils are present and stain sharply in absolute scorbutus (Figs. 2 and 9). No collagen or reticulum can be found in any attempt at repair during absolute scorbutus. As shown in Figure 2, the fibroblasts in such granulation tissue are never in close contact and very frequently they are separated by vacuoles outlined by very delicate acidophilic material which is often granular. This appearance of edema is also present in the *Gerüstmark* zone of the costochondral junction and in clusters of fibroblast-like cells on the internal surface of the resorbing cortical bone of ribs in scorbutus. In preparations stained with Mallory's connective tissue stain there is often a faint bluish coloration to the cytoplasm of fibroblasts in organizing blood clots in scorbutus and to the peripheries of the vacuoles between cells. The appearances indicate that in absolute scorbutus fibroblasts are responsible for the formation of an extracellular liquid. The fibroblasts (Fig. 2) themselves are vacuolated usually at their extremities, particularly at points of divergence of fibroglia fibrils, and a frequent picture is that of fibroblasts with spongy cytoplasm whose boundaries are delineated only by the aid of the fibroglia fibrils upon their surfaces. These appearances suggest that the contents of these vacuoles are discharged and accumulate to some degree between the cells. It is to be remembered that these observations apply to cells in regions remote from blood vessels and lymphatics, so that transudation as a source of edema is probably excluded. There can be little ground for denying that the extracellular liquid has its source in the cytoplasmic vacuoles. The vacuoles may be evidence of degeneration or the consequence of excessive secretion of an abnormal cell product; this study supports the latter interpretation.

The presence of collagen following administration of orange juice (10 to 15 cc. daily) was studied at 24, 48, 72 and 96 hour periods. For the sake of brevity the term collagen is used to include the material first deposited around fibroblasts in repair, including the intercellular material first laid down in the formation of bone. It includes so-called reticulum (argyrophile fibrils), as determined by silver impregnation methods, as well as the material having the normal staining properties of collagen with the methods employed in this study. Collagen and reticulum were found around cells, often completely isolated, far from blood vessels and preëxisting normal

geneous state was of short duration, though best seen on the second day when collagen deposition was rapid; no extensive zones of it were found. It was intimately applied to the cytoplasm of the cell body or to fibroglia fibril extensions beyond apparent limits of the cell. Its site of formation in relation to the cell body was always external to fibroglia fibrils. It was present about cells when no reticulum or argyrophile fibrils could be demonstrated.

Next in sequence was the appearance of delicate fibrils coincidently with the appearance of reticulum, or argyrophile fibrils (Figs. 5 and 6). With isolated, rounded or ovoid cells the direction of the fibrillary collagen, as well as that of argyrophile fibrils, was concentric to the cell body; if the cells were processed the fibrils paralleled the cell processes and fibroglia fibrils. In cell clusters the fibrils coursed irregularly between cells and it was impossible to associate any group of fibrils with a definite cell (Fig. 7).

Early in recovery from scorbutus, up to 72 hours (Fig. 6), the reticulum method gave the appearance of there being exceedingly delicate black fibrils embedded in a homogeneous collagen matrix, producing the effect of broad bands of black reticulum. This microscopic sequence may be explained as fibril formation in a homogeneous material. More mature collagen in later periods of recovery does not impregnate with silver. The first detectable argyrophile material appears in the form of black granules at the limit of microscopic resolution, arranged in rows, always somewhat tortuous, never straight. Identical sequences were found in the *Gerüstmark* zone of the ribs and in the resumption of osteoid deposit in the endochondral formation of bone (Fig. 8) and upon the inner ("endosteal") and outer (periosteal) deposition of osteoid.

The conclusion is unavoidable that the earliest formed collagen fibrils are identical with reticulum and that the property of impregnating with silver is due to delicacy of the fibrils, a conclusion reached by Mallory and Parker [5] from a study of human tissues and tumors, by Alfejew [6] from the study of mammalian embryos, and by Maximow [7, 8] from the study of rabbit tissue plasma cultures.

In the resumption of bone formation in recovery from scorbutus sequences identical to those described above take place, and the first deposit of intercellular material cannot be differentiated from that which forms in organizing blood clots and in other regions not destined to ossification.

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The study of bone resorption in regions of most marked osteoporosis in absolute scorbutus offers convincing evidence that some liberated bone cells survive and increase in size and acquire appearances usual to fibroblasts. Fibroglia fibrils can be seen in enlarged bone canaliculi and to extend into the enlarged marrow cavity. Traces of matrix extending inward from the cortical bone lie parallel to fibroglia fibrils. The lacunae in which bone cells lie are enlarged (Fig. 9). These facts are mentioned because they are some of the observations that indicate resorption of bone matrix and its collagen constituent is dependent upon the activity of those cells that under other conditions are concerned in its formation. They also indicate that the fibroglia fibrils are conductors of the agents concerned in resorption as well as in the deposition of matrices, and that fibroglia fibrils may have a cytoplasmic vesture. In recovery from scorbutus (Fig. 10) prompt formation of intercellular substance at first indistinguishable from collagen by reticulum staining methods and the usual connective tissue stains took place. As in the case of collagen formed in the organization of blood clots the deposit in bone formation was at first homogeneous, then fibrillary, and its distribution determined by cells and their processes. Studies which will not be presented here support the belief in the existence of a homogeneous material enveloping the collagen fibers in the completed bone matrix.

Summary and Discussion

The organization of blood clots during the state of absolute scorbutus in guinea pigs is a process in which fibroblasts become widely separated from their sources and from blood vessels, capillaries and lymphatics. Fibroblasts may also be found separated from one another, migrating and dividing as in tissue plasma cultures, following strands of fibrin and penetrating masses of blood corpuscles in regions where stainable threads of fibrin are absent. Collagen does not appear either as reticulum or in more easily demonstrable form until recovery is induced by the administration of an antiscorbutic. Under the conditions of these experiments the exact situation of reticulum and collagen formation has been determined.

Collagen appears first as a homogeneous material in which argyrophile or reticulum fibrils promptly appear. Coincidently with the appearance of the argyrophile fibrils the stains in common usage for

demonstrating collagen show the presence of collagen fibril bundles. The distribution of the collagen is dependent upon the form of the cell and with isolated cells is confined to zones immediately adjacent to the cell body and its processes, including the entire length of fibroglia fibrils.

The course or direction of the collagen and argyrophile fibrils is parallel to surfaces of the fibroblast and its processes. Because of this arrangement parallel to the cell and its processes, and never radiating, fibril formation must be influenced by factors not present in the formation of fibrin strands from plasma, and speculation is suggested concerning a molecular alignment previous to the development of fibrils. Further speculation suggests that the pattern of collagen fibrils formed rapidly in groups of cells which are without processes is influenced by many cells as by a resultant of forces acting in the homogeneous or amorphous stage of collagen formation. Where the processes and fibroglia fibrils of fibroblasts interweave the appearances are that each cell is accompanied by collagen fibrils in parallel arrangement to its ramifications.

Careful study of regions containing fibrin in experimentally produced clots and in regions of spontaneous injury, particularly at the line of infraction in ribs, yielded no suggestion of transformation of fibrin into collagen. The presence of fibrin does not modify the arrangement of collagen about the fibroblasts, which is conclusive evidence that fibrin does not directly contribute to the formation of collagen.

Baitsell,[9–12] both in tissue plasma cultures and in the formation of tuberculous tissue in the guinea pig testis, has presented evidence for the transformation of fibrin and exudate into collagen. His material was so different from that of the present study that it is impossible to exercise critical judgment in comparison of conclusions. It may be said that the conditions in tuberculous testes of guinea pigs are exceedingly complex, as compared to the recovery phenomena in organization of blood clots in scorbutus.

Nageotte,[13] in many types of experiments, concludes that fibrin is transformed into collagen, but also states that other albuminoid substances, including the protoplasm of dead cells, may be precursors of collagen.

In embryos a common and well substantiated origin of collagen is "a transparent, gelatinous, cell-free, ground substance which, in

general, pervades the embryonic body from very early stages of development."[15] The recent papers of Baitsell,[14, 15] Harrison,[16] and Alfejew [6] all express the opinion that fibrils form in this ground substance independently of the immediate proximity of cells. Maximow and Bloom [8] describe spiral argyrophile fibrils formed in tissue cultures remote from cells. This observation seems to have been made by Bloom, as it does not appear in Maximow's publication [7] based upon the same material.

An early conception of the origin of collagen fibrils was the direct modification of the cytoplasm of the surface of the fibroblast and its processes, held by Hansen,[17] and Mall,[18] and other noted investigators. The most substantial recent support of this view is that of M. R. Lewis [19] from tissue culture studies. According to Lewis the fibrils appeared in cultures after 24 hours growth, "as slightly more refractive lines within the cytoplasm of the individual cell" and later became gathered into bundles outside of the cell to form slender fibers. Mitochondria were described as being occasionally carried by fibrils to points outside of the cell, though not concerned in fibril formation, as has been held by Meves,[20] and others. The conclusions of this impressive paper cannot be reconciled with those of the present study. A further divergence is Lewis' conclusion that the vacuoles which form in fibroblasts are not concerned in fibril formation, as first suggested by Péterfi [21] in the study of argyrophile fibrils in epithelial cells of the amnion of fowl embryos.

Whether or not the vacuoles that form in fibroblasts in the scorbutic guinea pig have a counterpart in fibroblasts in granulation tissue under normal conditions has not been determined. Their presence in scorbutus may be regarded as pathological, but also as due to an exaggeration of a normal process, and in all probability as the source of the extracellular liquid which is so conspicuous in certain regions in experimental scorbutus and which may represent a liquid precursor of collagen, as suggested by the rapid appearance and large volume of intercellular material in the recovery from scorbutus.[1]

In the above discussion reference has been made only to papers with a bearing upon observations made in the present study. Excellent reviews covering the literature of collagen formation are to be found in Lewis' paper [19] and by Maximow in Möllendorff's Handbuch.[22] Relevant to the subject, as concerning intercellular substances, are the recent articles with exhaustive reviews of the

literature on cartilage by Schaffer,[23] and on bone by Weidenreich,[24] also in Möllendorff's Handbuch.

Consideration of the three theories held in explanation of collagen fibril formation — (1) the intracellular by transformation or metaplasia of the surface cytoplasm (ectoplasm) with or without the participation of mitochondria; (2) the origin in an extracellular amorphous ground substance secreted by cells, and (3) the transformation by enzymes of extracellular material not produced by fibroblasts, such as fibrin or other forms of exudate and albuminoid materials from dead cells — shows compatibility of only the second with the results of the present study. In embryos large amounts of amorphous precollagen are formed and fibril formation apparently takes place in regions remote from cells. In Maximow's tissue cultures the first evidence of intercellular materials was the formation of argyrophile fibrils, apparently not necessarily in close contact with cells and extending into the medium without relation to cell processes.

In recovery from scorbutus the collagen deposit is restricted to the immediate vicinity of fibroblasts. It is at first amorphous. Argyrophile fibrils appear first in the earliest deposited material; this can be best seen in the resumption of bone matrix formation in endochondral bone formation (Fig. 8). About fibroblasts the collagen distribution is definitely related to the cell body and its processes, including the fibroglia fibrils. The study of resorption of bone and the shapes assumed by released bone cells indicates that the fibrils of the latter are identical with fibroglia fibrils and that they in some manner are active in the resorption of bone matrix. The distribution and directions of collagen fibrils in recovery from scorbutus are also influenced by fibroglia fibrils. In addition to possible mechanical functions fibroglia fibrils are conductors of agents presumably having reversible enzyme activities.

The writer's evaluation of the conclusions drawn from this study is influenced by unpublished studies of sequences in resumption of growth of bone in recovery from rickets, scorbutus and vitamin A deficiency. In each instance the reparative processes were identical with the normal sequences that the deficiency had suspended, but they proceeded, in their early stages, at a rate far beyond that of normal growth. This is probably true of recovery phenomena from all vitamin deficiencies and seems definitely so in the resumption of collagen formation by fibroblasts in the present study.

CONCLUSIONS

1. Fibrin and other preformed materials do not contribute to collagen formation in repair by organization.

2. Collagen and reticulum represent physical differences of the same material.

3. Collagen is the product of secretory activity of fibroblasts, and its alignment and distribution are determined by the shape of the cell and its processes, including fibroglia fibrils.

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DESCRIPTION OF PLATES

PLATE 112

FIG. 1. Repair by avascular organization in absolute scorbutus. A strand of fibrin crosses the upper right-hand corner. Most of the cells in this field have probably emigrated from connective tissue surrounding the nerve shown in the lower border. Most of the fibroblasts are separated from one another. Five mitotic figures are in this field. Guinea pig operated upon on 23rd day of vitamin C free diet; killed on 30th day of deficiency. Initial weight 315 gm., maximum weight 360 gm., and final weight 204 gm. Modified Giemsa stain. × 125.

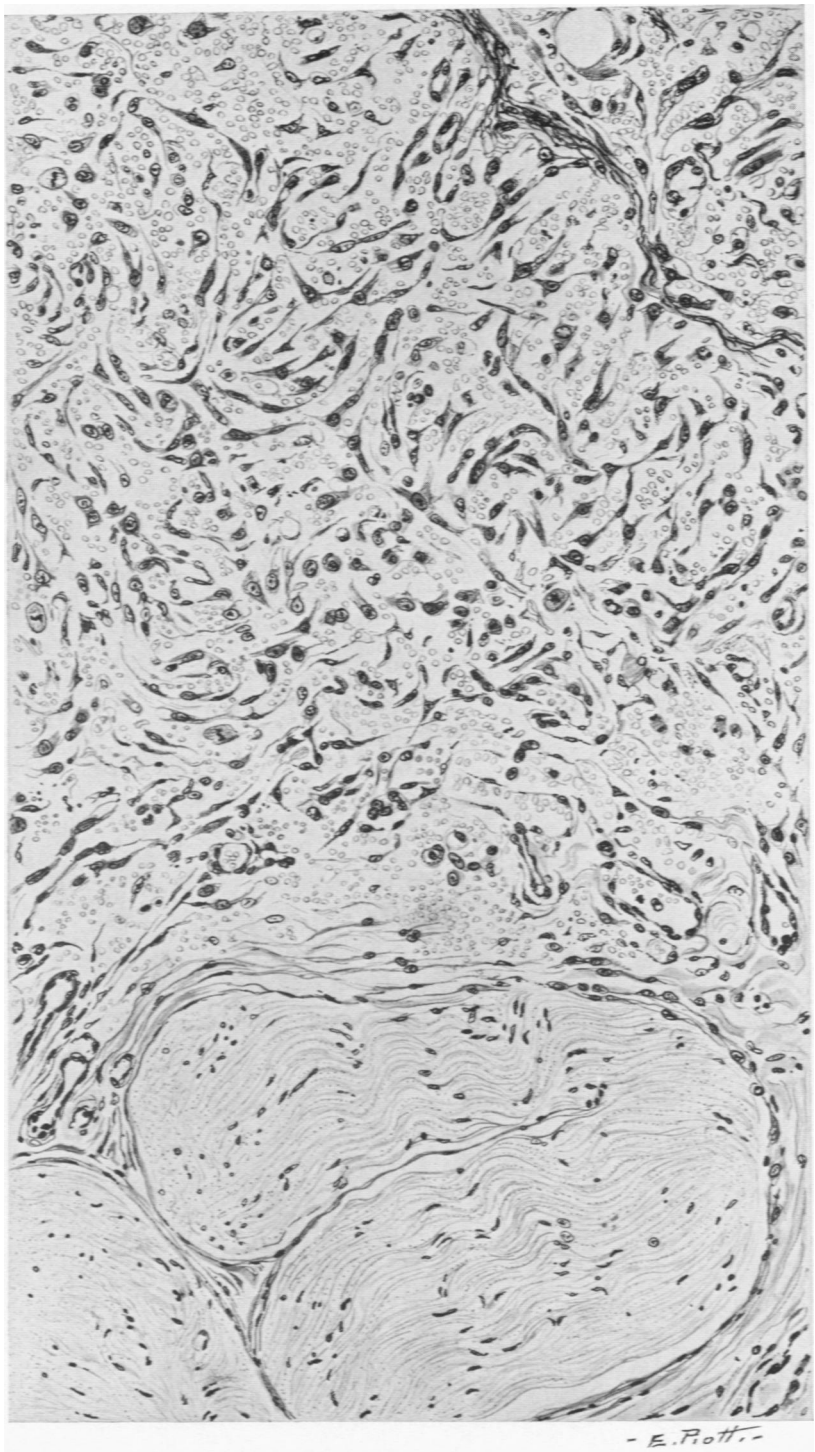

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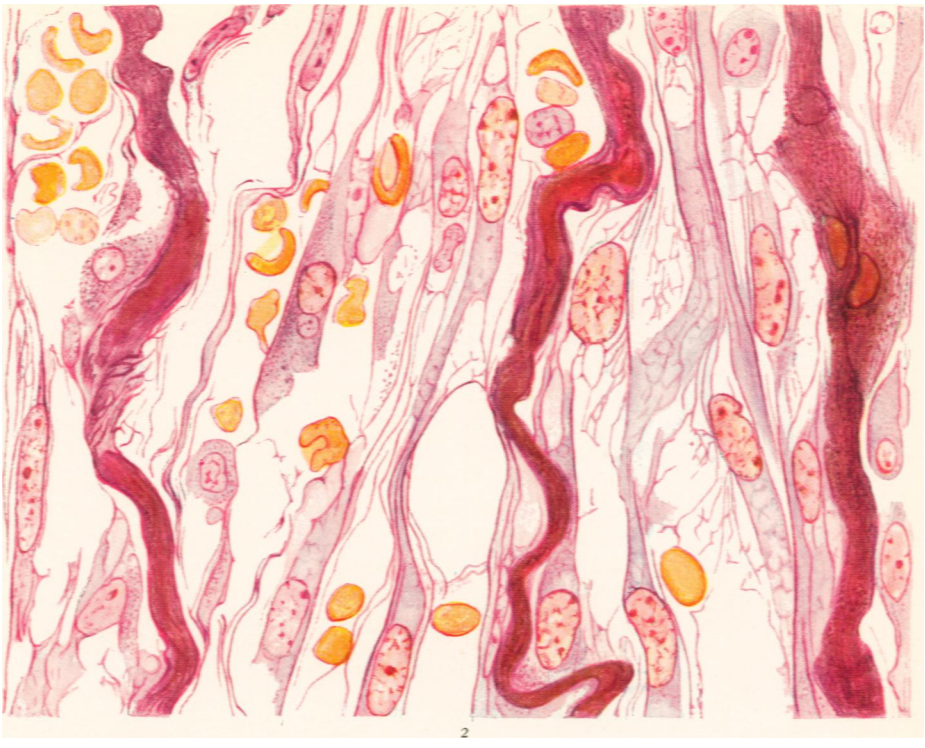
PLATE 113

FIG. 2. Fibroblasts in relation to fibrin strands in organization in absolute scorbutus. Fibroglia fibrils are prominent. Note vacuoles in cytoplasm of the fibroblasts and extracellular vacuoles described in the text. No stainable collagen, including argyrophile fibrils, present. Guinea pig operated upon on 23rd day of deficient diet; killed on 33rd day of the deficiency. Initial weight 400 gm., maximum weight 446 gm., final weight 320 gm. Mallory's connective tissue stain. × 1000.

FIG. 3. To illustrate the collagen formed in organization tissue during a 40 hour period of recovery from absolute scorbutus. The region illustrated is deep in the blood clot. Note homogeneous appearance of the collagen surrounding cells in contact with fibrin in left third of the field; fibrillation of collagen is apparent elsewhere. Other preparations stained by Foot's method showed argyrophile fibrils in small numbers and corresponding to the fibrils illustrated. Guinea pig operated upon on 23rd day of deficient diet; orange juice administered on afternoon of 28th day; killed on morning of 30th day of the experiment. Initial weight 357 gm., maximum weight 395 gm., final weight 301 gm. Mallory's connective tissue stain. × 1000.



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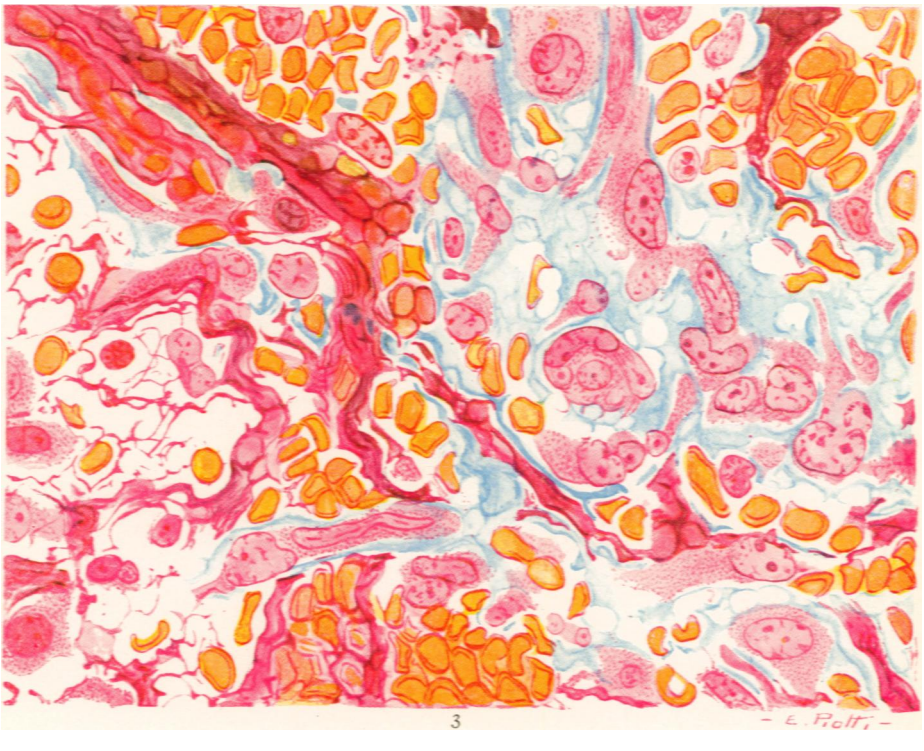

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PLATE 114

FIG. 4. To show collagen in relation to an isolated cell deep within the blood clot. Recovery period of 72 hours. Guinea pig operated upon on 23rd day of deficient diet, orange juice first given on 31st day of experiment. Initial weight 395 gm., maximum weight 418 gm., final weight 288 gm. Mallory's connective tissue stain. × 1000.

FIG. 5. Recovery period of 72 hours; same animal used for Fig. 4. A field from a group of cells deep within the clot, illustrating collagen deposited by cells that have penetrated fibrin strands. Mallory's connective tissue stain. × 1000.

FIG. 6. Recovery period of 72 hours. This field corresponds to that of Fig. 5 and is from the same block at a slightly different level. The argyrophile fibrils have formed and their distribution in relation to the cells is the same as that of the collagen. Foot's modification of Bielschowsky-Maresch silver impregnation method, followed by Mallory's connective tissue stain. × 1000.

FIG. 7. To illustrate collagen formed by a cluster of cells during a 96 hour repair period. Guinea pig operated upon on 23rd day of deficient diet; orange juice administered from 31st day to 35th day inclusive; killed on 36th day of experiment. Mallory's connective tissue stain. × 1000.

FIG. 8. To illustrate resumption of endochondral bone formation. Rib, recovery period of 40 hours duration. Preparation from the same guinea pig used for Figs. 3 and 10. To illustrate newly formed argyrophile fibrils in presence of homogeneous collagen representing the first stage in formation of the intercellular substance of bone. Argyrophile fibrils have formed in the amorphous collagen first deposited by the immature osteoblasts. Staining technique same as that of Fig. 6. × 430.

FIG. 9. "Endosteal" surface of rib of guinea pig in absolute scorbutus. To illustrate details of bone resorption described in the text. Note the appearances of cells indistinguishable from fibroblasts which in recovery from scorbutus become osteoblasts. The yellow mottling of the bone is characteristic of advanced scorbutus, when stained by Mallory's method. Note the continuity of fibroglia fibrils emerging from the cortex with the fibroblasts in the cavity of the bone. Mallory's connective tissue stain. × 1000.

FIG. 10. To illustrate early "endosteal" bone formation during a 40 hour recovery period and relation of the collagen to cell shapes and fibroglia fibrils. Rib, from guinea pig used for Figs. 3 and 8. Mallory's connective tissue stain. × 1000.

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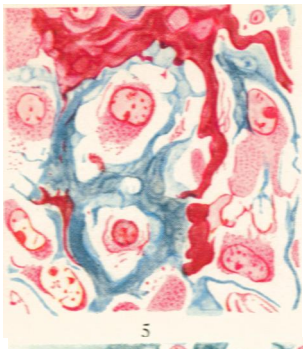

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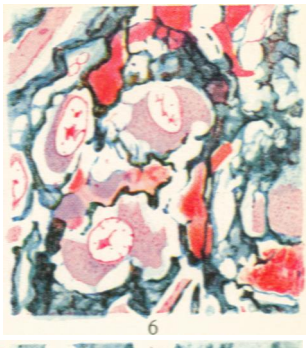

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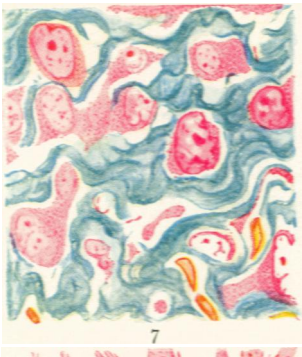

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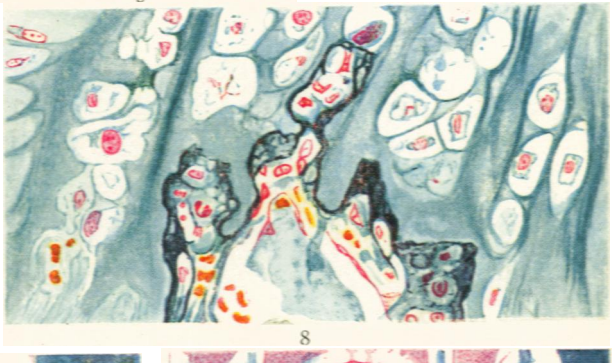

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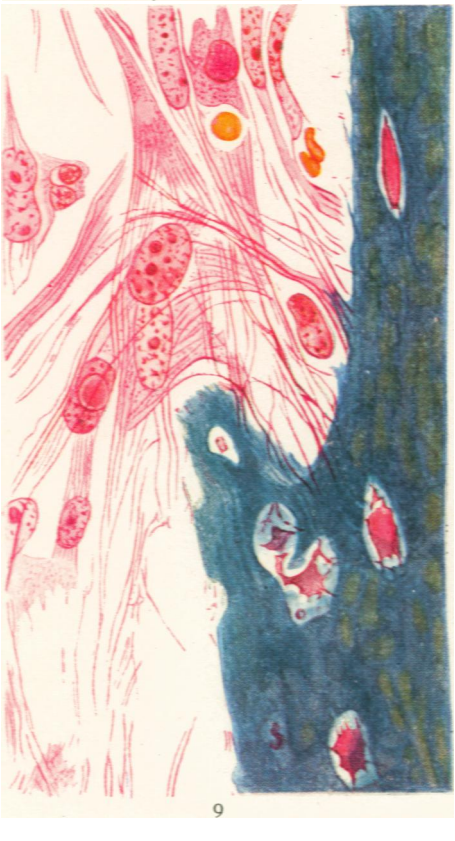

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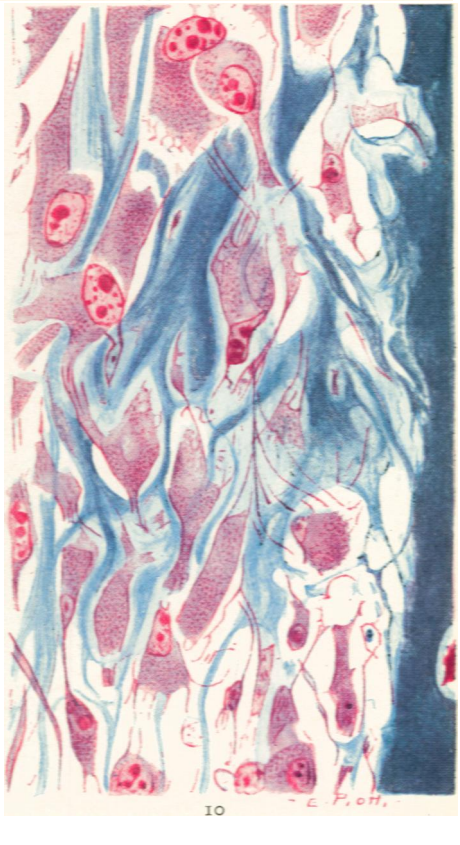

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