THE FORMATION OF COLLAGEN FIBERS BY THE ACTION OF HEPARIN ON SOLUBLE COLLAGEN: AN ELECTRON MICROSCOPE STUDY*

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PLATES 3 AND 4

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Recent experiments by Highberger, Gross, and Schmitt (1) indicate that components of the ground substance may play a role in the formation of collagen. These authors have produced fibers *in vitro* by adding mucoprotein and mucopolysaccharides to solutions of collagen. On the basis of examination of these reconstituted fibers with the electron microscope, three types are described: (*a*) fibers showing a periodicity of 650 A, characteristic of collagen, (*b*) long-spacing (LS) fibers with an axial period ranging from 2000 to 3000 A, and (*c*) "unstructured" fibers, showing no cross-striations. These different types of fibers were obtained by utilizing varied concentrations and combinations of collagen solutions and fibril-precipitating agents. Highberger *et al.* (1) have employed citrate extracts and acetic acid filtrates of collagen, together with serum mucoprotein, hyaluronic acid, chondroitin sulfate, and heparin in their experiments.

The constant presence of mast cells in connective tissue and areas of fibrosis (2, 3), suggested to us that these cells, by the action of the heparin which they produce (4–7), may play a role in the formation of collagen. In the present study, heparin was added to soluble collagen, and the fibers which resulted were viewed with the electron microscope. A survey of pathological material was also done in order to confirm the presence of mast cells in foci of fibrosis.

Methods

Dura mater was obtained at autopsy from newborn infants within a few hours after death. The dura was freed of blood by making incisions in blood vessels visible in the gross, and washing it repeatedly in distilled water. A 1 gm. sample was cut into small pieces and ground in a Waring blendor for 5 minutes in 200 ml. of acetic acid (0.04 per cent) in distilled water. The material was cooled intermittently during the grinding procedure. The resulting mixture was allowed to stand at 6° C. for 18 to 24 hours. It was then centrifuged for 30 minutes in an International centrifuge at 3000 R.P.M. The supernate was recentrifuged in a Sorval-type SSI angle head centrifuge at 13,500 R.P.M. for 45 minutes, in a cold room. The uppermost

* All electron micrographs were taken by Dr. Keith R. Porter, of The Rockefeller Institute for Medical Research, to whom we are deeply grateful.

fifth of the slightly viscous and opalescent supernatant solution thus obtained was employed in the experiments. The total nitrogen content of the solution averaged 0.05 per cent.

Powdered heparin[1] was dissolved in 0.04 per cent acetic acid in distilled water or in 1 per cent NaCl, and dilutions were made of it which ranged from 1–100 to 1–80,000. Equal volumes of the collagen solution were added to the heparin solution, giving a final series of heparin dilutions of 1–200 to 1–160,000.

Suspensions of the precipitated fibers were diluted with an equal volume of distilled water, placed on formvar-coated grids, and allowed to dry in air. The specimens were shadowed with chromium at an angle of 12° and examined under a Philips model EM 100 electron microscope.

Aggregates of heparin-precipitated fibers were obtained in pellets by centrifugation at 2500 R.P.M. in an International centrifuge for 15 minutes. These were wrapped in filter paper, fixed in Zenker's fluid, sectioned, and stained with Masson's trichrome, Van Gieson's stain, and Laidlaws' reticulum stain. Tissue sections which showed various examples of fibrosis were prepared from human autopsy and surgical specimens, and stained with Giemsa and toluidine blue stains for mast cells.

RESULTS

The addition of heparin to an acetic acid solution of collagen results in the immediate formation of fibers visible in the gross. The appearance of fibers is accompanied by slight clearing and an apparent diminution in the viscosity of the collagen solution. As the mixture is gently agitated, the delicate fibers form a loose meshwork, which tends to sink to the bottom of the test tube. Examination of the fibers under a polarizing microscope showed them to be doubly refractile.

Dilutions of heparin in distilled water as high as 1–80,000 produced immediate formation of fibers with prompt precipitation. The total amount of formed fibers was approximately the same with heparin dilutions of 1–200 to 1–20,000, and slightly less with dilutions of 1–40,000 and 1–80,000. The rapidity of fiber formation was practically simultaneous with all concentrations of heparin that gave a positive result. Similar attempts to reconstitute collagen from acetic acid extracts of dura mater from adults or rat tail tendon were unsuccessful.

Observations with Other Chemical Compounds

Paritol (sodium polyanhydromannuronic acid sulfate), a substance which is related to heparin chemically, produced precipitation of fibers from acetic acid solutions of collagen. The action of paritol was observed to be effective with dilutions of this substance in distilled water as high as 1–40,000. The paritol precipitation closely resembled that induced by heparin in the rapidity of fiber

[1] Heparin-sodium salt 100 units per mg., made by the Connaught Medical Research Laboratories of Toronto, Canada, was used in the experiments. Similar results were obtained with heparin-sodium solution Abbott 1000 U.S.P. units per cc. of isotonic saline, in spite of the presence in this preparation of 0.5 per cent phenol which may act as a protein denaturant.

formation. There was also a gross and microscopic similarity in the type of precipitate which was formed by the action of heparin and paritol.

It is known that various concentrations of NaCl as well as alterations in pH will result in the reconstitution of fibers from acetic acid solutions of collagen. Vanamee and Porter (8), utilizing electron microscopy, have observed cross-striated collagen fibers having an axial period averaging 640 A in these preparations of NaCl-reconstituted collagen fibers. They also have demonstrated that fibers precipitated by higher concentrations of NaCl show no periodicity. In our experiments the pH ranged from 3.2–3.8 before and after the addition of heparin, a zone at which spontaneous reconstitution of collagen fibers is not observed. Vanamee and Porter reconstituted collagen fibers from acetic acid solutions of collagen by raising the pH to 4.8–7.8. Although fiber formation was observed upon the addition of 1 or 2 per cent NaCl to our solutions of collagen, this reaction did not occur until the preparations had remained at room temperature for 12 to 24 hours. Moreover, solutions of heparin in distilled water were as effective in precipitating fibers as saline solutions of heparin.

Attempts were made to produce precipitation of fibers by adding chemical components or structural units of ground substance to acetic acid solutions of collagen. Negative results were obtained with the following compounds: glucuronic acid, glucosamine HCl, K ethyl sulfate, and *N*-acetyl glucosamine, each in concentrations of 10 mg. per cc. in distilled water. According to Schmitt (1, 9) hyaluronic acid produces an immediate precipitate of fibers devoid of structure, but in the presence of 1 per cent NaCl, LS as well as unstructured fibers were obtained. We obtained similar results with potassium hyaluronate from vitreous humor. Observations with the electron microscope were not made of the fibers precipitated with hyaluronate from the collagen of infant dura, but K hyaluronate–precipitated fibers from acetic acid solutions of rat tail tendon were examined, and they showed no periodicity.

Electron Microscopy

The heparin-precipitated fibers were found to be elongated filamentous structures which varied from approximately 500 to 8000 A in width (Fig. 1). The fibers frequently exhibited a fine longitudinal structure (Fig. 6), appearing as bundles of delicate fibrils. The latter were sometimes visible individually in the surrounding medium (Fig. 6), and ranged in size from those which could barely be resolved at a magnification of 50,000 diameters (that is to say about 50 A across) to others that were 240 A or more in width. The widest fibers appeared to be those with the most loosely packed bundles of delicate fibrils (Figs. 1 and 2). No cross-striations or axial periodicity was observed in this type of broad, loosely organized fiber.

However among those fibers which were more compact there were segments

which showed a distinct periodicity of approximately 640 A (Figs. 1 to 5). This finding demonstrates conclusively that many of the fibers formed by the heparin precipitation were true collagen. It is assumed that those fibers which did not show periodicity had failed to organize properly. This may have been due in part to the great rapidity with which they formed, or to impurities in the original collagen solution. Fig. 7 shows the only collagen fiber which was found during the examination of six control specimens of the original collagen solution as such, from which one may conclude that the latter had been virtually completely freed of undissolved fibers by the high speed centrifugation.

In many instances, large numbers of formed fibers were encountered which were of approximately equal widths, and of similar texture. These fibers characteristically showed a periodicity of 640 A, but only throughout short segments (Fig. 5). Frequently structureless zones were observed to alternate with segments showing periodicity. Very occasionally, fibers of the long-spacing type were recognized (Fig. 8).

Reactions with Histological Stains for Collagen

The heparin-precipitated fibers were found to give typical staining reactions for collagen with Masson's trichrome and Van Gieson's stains. Except for variation in the intensity of staining, all fibers stained in this manner. One can assume therefore that the specificity of collagen stains does not depend upon periodicity in fibers, since these stains do not distinguish between non-striated and classical collagen fibers. The positive staining reaction of the former suggests that they are truly collagenous, in spite of their structureless appearance under the electron microscope.

Laidlaw's reticulum stain demonstrated some fibers which were strongly argyrophilic, but most fibers remained unstained. We were unable to establish any correlation between argyrophilia and the presence of periodicity in the heparin-precipitated fibers.

Presence of Mast Cells in Foci of Fibrosis

Previous reports (2, 3) describe the presence of mast cells in sites where collagen exists or is being produced. Janes and McDonald (2) examined human tissues and found that mast cells were present in connective tissue wherever it was studied. Mast cells were found in increased numbers in tissues which were the site of chronic inflammation, as well as in various tumors. We recognized mast cells in varying numbers in fibrous tissue in the following conditions: cirrhosis of the liver, chronic cholecystitis, chronic synovitis, chronic ulcer of the duodenum, tuberculosis of the kidney, and chronic pancreatitis. Tumors in which mast cells were also seen were lipomyxosarcoma, scirrhous carcinoma of the breast, sclerosing hemangioma of skin, retroperitoneal fibrosarcoma, and fibroadenoma of the breast. Mast cells were found to be especially

numerous in sections of chronic duodenal ulcer, chronic synovitis, and chronic cholecystitis, averaging from three to eight per high power field.

DISCUSSION

The current concepts of the mechanism of collagen formation are discussed in detail in recent excellent review articles (10–12). Although it is generally believed that collagen is formed by the action of fibroblasts there is disagreement concerning the mechanism of fibrillogenesis and the role of the ground substance in fiber formation.

Of special interest and importance are recent studies by Porter (13) of the formation of collagen fibers in tissue cultures of fibroblasts. By means of observations with the electron microscope, he showed that collagen fibrils are formed at the surface of fibroblasts. He postulates that the ectoplasm of these cells may show organization into fibrils beneath the cell membrane, or that components of the ectoplasm may pass through the cell membrane and polymerize into definite fibrils on the surface of cells. Moreover, Porter has observed an interfiber substance in tissue cultures of fibroblasts which he believes may be a mucoprotein component of ground substance.

These experiments which conclusively demonstrate the formation of collagen fibers in tissue cultures of fibroblasts appear to argue against the hypothesis that mast cells and heparin are important in the production of collagen. However, it is possible that mast cells occur as contaminants in tissue cultures of fibroblasts. It is conceivable, therefore, that even small numbers of mast cells under these conditions could produce sufficient heparin to influence collagen fiber formation, in view of the ease with which heparin dissolves in aqueous solutions, and the efficacy with which small traces of heparin have precipitated collagen in our experiments.

Indirect evidence whch may be taken to imply that heparin favors the formation of collagen has been presented by McCleery, Schaffarzick, and Light (14). They found that the speed and effectiveness of tissue repair following experimental burns were enhanced by heparin, and the healing time was shortened by the administration of this substance.

Meyer and Rapport (15) in a description of the mucopolysaccharides of the ground substances of connective tissue, mentioned heparin as a possible sixth mucopolysaccharide of ground substance. They believe that heparin has not been identified in ground substance either because its concentration is too low, or because it may be lost in the isolation procedure.

Chondroitin sulfate, a mucopolysaccharide which is closely related to heparin, has been found by Schmitt to precipitate LS and unstructured fibers from solutions of collagen (9). The interrelationship between the three types of chondroitin sulfate (A, B, C), as well as that between these substances and heparin remains to be clarified. Klemperer (10) pointed out that although most observers believe that compo-

nents of the ground substance are formed by fibroblasts, actual conclusive proof is not available. The observations by Gersh and Catchpole (16) of cytoplasmic glycoprotein in fibroblasts are based solely on histochemical methods, and do not prove that this substance is the precursor of mucopolysaccharides of ground substance.

Meyer's theory of fibrillogenesis (17–19) proposes that fibroblasts produce a globular native protein precollagen which is precipitated and denatured onto the surface of polysaccharide fibrils by local acid production by the cells, presumably by glycolysis. The mucopolysaccharides which are initially in the fibers are then removed by enzymatic digestion.

The experiments reported by Bensley (20) show that following the injection of the spreading factor of testicular extracts into the skin of mice, there are fibroblastic activity and a reduction in the number of mast cells. After 4 days, evidence of new fiber formation appeared, and concomitantly there was an increase in the number and granulation of mast cells. Bensley concluded that although the role of the mast cell in this process is not clear, her experiments suggest that the mast cells may be concerned with the segregation of polysaccharides and perhaps associated enzymes as substrate-enzyme complexes.

These current theories of the mechanism of collagen fiber formation are compatible with our current demonstration of the efficacy of heparin in inducing the formation of collagen fibers *in vitro*. The relative importance of heparin and the other components of the ground substance in fibrillogenesis remains to be clarified.

SUMMARY

Collagen fibers have been formed *in vitro* by the action of heparin on solutions of collagen.

Heparin was found to be effective in inducing collagen fiber formation when present in concentrations as low as 1–80,000.

It is postulated that mast cells, by virtue of the heparin which they produce, may play a role in the formation of collagen.

BIBLIOGRAPHY

1. Highberger, J. H., Gross, J., and Schmitt, F. O., *Proc. Nat. Acad. Sc.*, 1951, **37,** 286.
2. Janes, J., and McDonald, J. R., *Arch. Path.*, 1948, **45,** 622.
3. Staemmler, M., *Frankf. Z. Path.*, 1921, **25,** 391.
4. Jorpes, J. E., Heparin, London, Oxford University Press, 1946.
5. Holmgren, H., and Wilander, O., *Z. mikr.-anat. Forsch.*, 1937, **42,** 242.
6. Jorpes, J. E., Holmgren, H., and Wilander, O., *Z. mikr.-anat. Forsch.*, 1937, **47,** 279.
7. Oliver, J., Bloom, F., and Mangieri, C., *J. Exp. Med.*, 1947, **86,** 107.
8. Vanamee, P., and Porter, K. R., *J. Exp. Med.*, 1951, **94,** 255.
9. Schmitt, F. O., personal communication.
10. Klemperer, P., *Am. J. Path.*, 1950, **26,** 505.

11. Ehrich, W. E., *Am. Heart J.*, 1952, **43,** 121.
12. Angevine, D. M., *Tr. 1st Conf. Connective Tissues,* Josiah Macy, Jr. Foundation, New York, 1950, 13.
13. Porter, K. R., *Tr. 2nd Conf. Connective Tissues,* Josiah Macy, Jr. Foundation, New York, 1951, 126.
14. McCleery, R. S., Schaffarzick, W. R., and Light, R. A., *Surgery,* 1949, **26,** 548.
15. Meyer, K., and Rapport, M. M., *Science,* 1951, **113,** 596.
16. Gersh, I., and Catchpole, H. R., *Am. J. Anat.*, 1949, **85,** 457.
17. Meyer, K., *Tr. 1st Conf. Connective Tissues,* Josiah Macy, Jr. Foundation, New York, 1950, 32.
18. Meyer, K., *Am. J. Med.*, 1946, **1,** 675.
19. Meyer, K., *Physiol. Rev.*, 1947, **27,** 335.
20. Bensley, S. H., *Ann. New York Acad. Sc.*, 1950, **52,** 983.

EXPLANATION OF PLATES

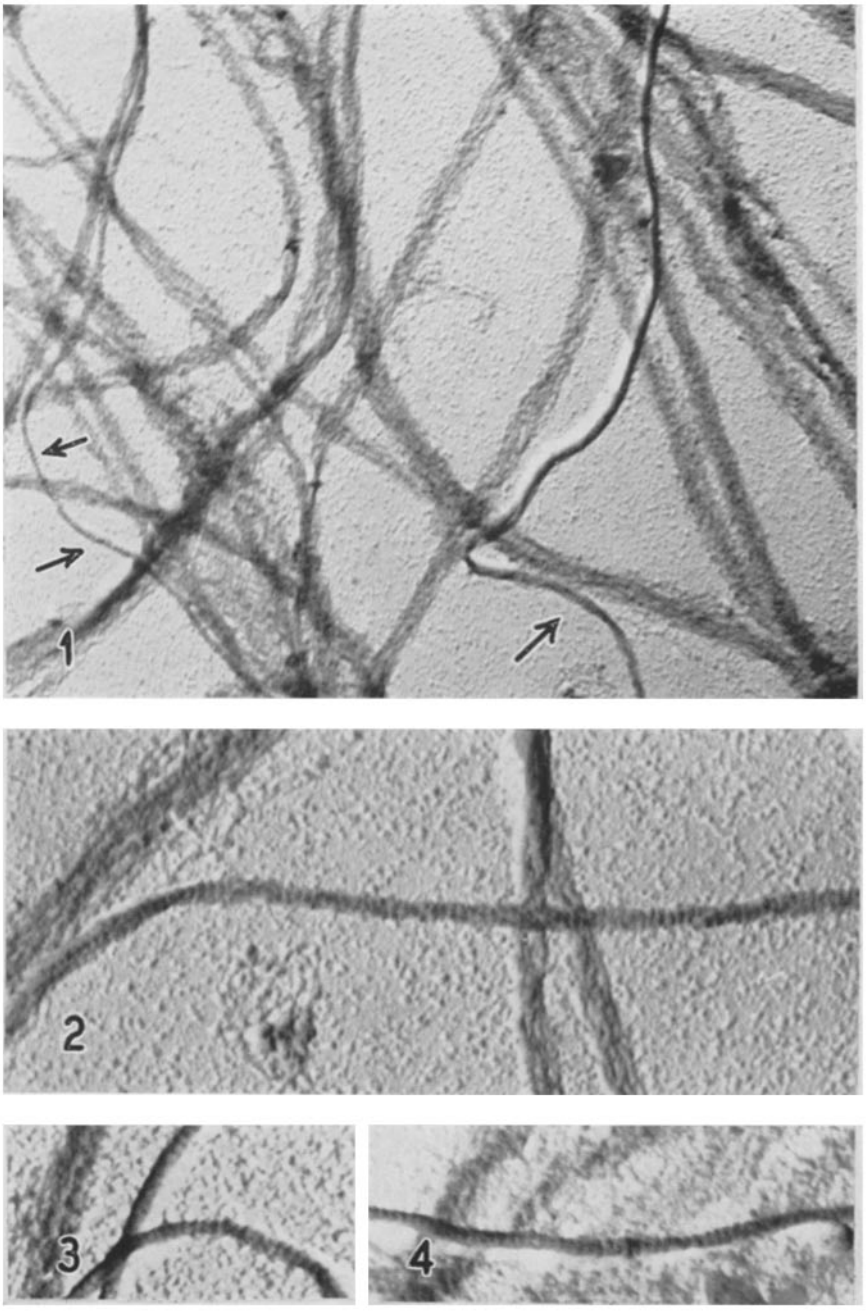
PLATE 3

FIG. 1. Heparin-precipitated collagen fibers. The fibers form a loose network. Note the differing diameter of the fibers, and a barely discernible periodicity (in the areas indicated by arrows). Shadowed with chromium. × 10,200.

FIG. 2. Collagen fibers formed by the addition of heparin to an acid solution of collagen. The long fiber shows cross-striations which have an axial periodicity of approximately 640 A. Shadowed with chromium. × 24,000.

FIG. 3. Two newly formed collagen fibers showing a periodicity of 640 A. Shadowed with chromium. × 24,000.

FIG. 4. Distinct axial periodicity of approximately 640 A in heparin-precipitated collagen fibers. Shadowed with chromium. × 24,000.



(Morrione: Formation of collagen by action of heparin)

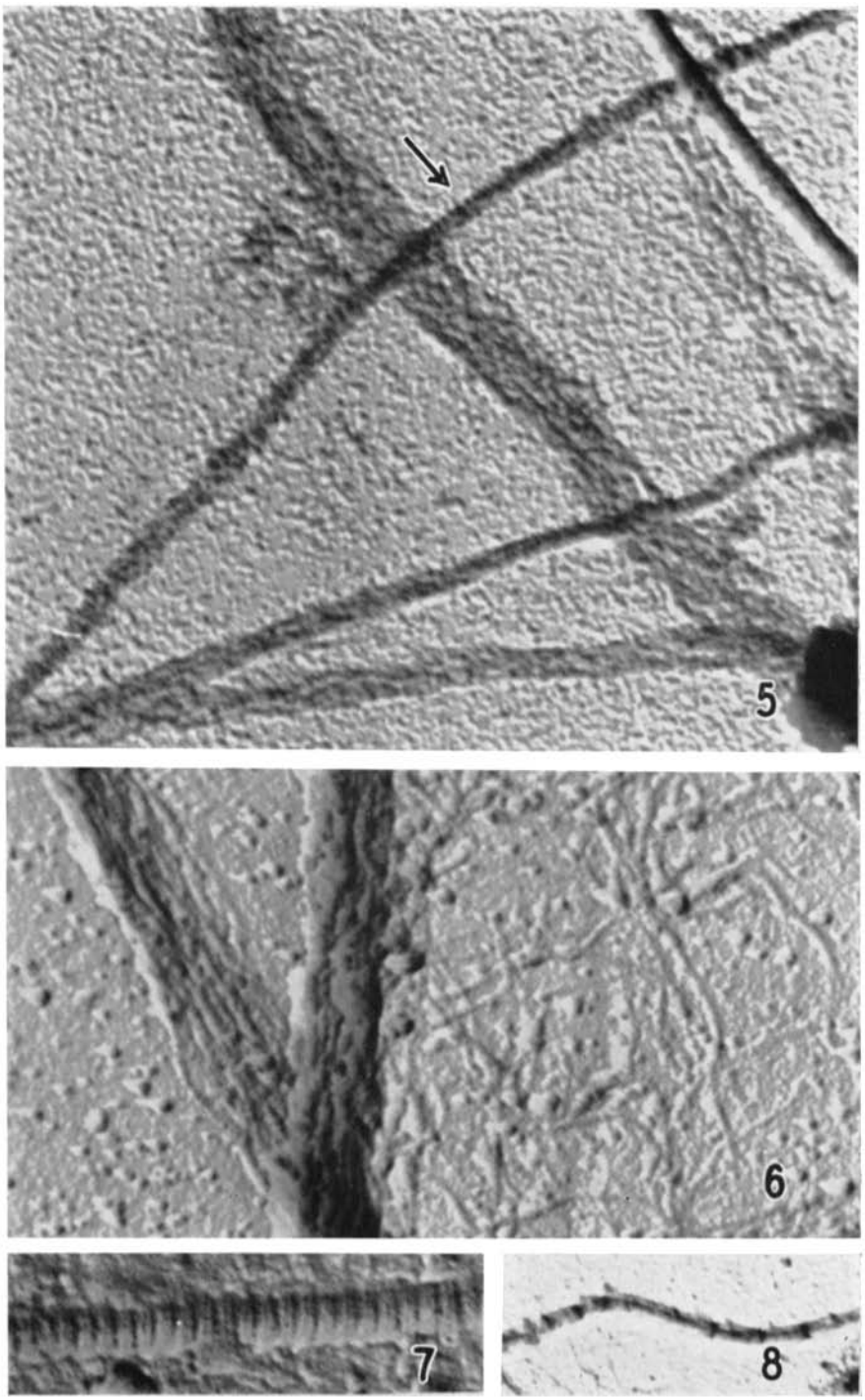
PLATE 4

FIG. 5. Group of heparin-precipitated fibers. Three fibers of similar appearance course diagonally across the micrograph. The arrow points to a segment of one which shows indistinct cross-striations with an axial periodicity of approximately 640 A. Shadowed with chromium. × 24,000.

FIG. 6. Chromium-shadowed preparation of fibers precipitated by heparin. The two large fibers show no cross-striations; they appear to be comprised of bundles of smaller fibrils of the type seen in the background. × 50,000.

FIG. 7. Collagen fiber which persisted in the collagen substrate as a contaminant. Contaminating fibers were rarely observed in the control collagen substrate, this fiber being the only contaminant which was observed in a series of six control specimens. Shadowed with chromium. × 50,000.

FIG. 8. Long-spacing type of fiber in heparin-precipitated collagen preparation. The periodicity varies from 1900 to 3500 A. Shadowed with chromium. × 24,000.



(Morrione: Formation of collagen by action of heparin)