of heparin. Using this method, the nature of the permeability activity of diluted serum was investigated and found to be caused solely by Pf/dil.

1. Kagen, L. J., Leddy, J. P., Becker, E. L., J. Clin. Invest., 1963, v42, 743.
2. Kagen, L. J., Brit. J. Exp. Path., 1964, v45, 604.
3. Mason, B., Miles, A. A., Nature, 1962, v196, 587.
4. Engelberg, H., Heparin, Thomas, Springfield, Illinois, 1963, 44.
5. Elder, J. M., Wilhelm, D. L., Brit. J. Exp. Path., 1958, v39, 23.
6. Ratnoff, O., Miles, A. A., ibid., 1964, v45, 328.
7. Miles, A. A., Wilhelm, D. L., ibid., 1955, v36, 72.
8. Smith, R. J., J. Clin. Invest., 1957, v36, 605.

Received February 19, 1965. P.S.E.B.M., 1965, v119.

Histoimmunological Study of Collagen During Tendon Fibrillogenesis. (30263)

R. E. MANCINI, J. BARQUET, M. A. PAZ,* AND O. VILAR
(Introduced by Hans Popper)
Departamento de Histologia, Facultad de Medicina, Universidad de Buenos Aires, Argentina

Although there is general agreement that the tropocollagen molecules originate in the fibroblast, the morphological evidence for intra- or extracellular formation of collagen fibers at the optical and cellular electron microscopic levels and the organization of the fibers is still controversial(1,2). Connective tissue extracts(3), an acetic acid extract of collagen(4), and also of soluble and insoluble fractions(5) are antigenic. These soluble and insoluble fractions in the collagen fibers of adult connective tissue have been visualized by immunofluorescence(6). Since, according to chemical studies of skin and granulomas, neutral salt soluble collagen has the properties of a precursor of both acid and insoluble collagen(7), a preliminary attempt is being reported to localize the soluble and insoluble fractions during collagen formation in the normal, the developing, and the injured tendon.

Material and methods. The purified neutral salt soluble and the insoluble fractions of collagen extracted from leg tendons of adult chickens were used as antigens. The preparation of these fractions, the schedule of the heterologous sensitization of adult rabbits, the periodic bleeding for gamma globulin determination, and the serological tests of the titers and the specificity of corresponding antibodies were previously reported(5) as were details concerning the separation of gamma globulin fractions, the conjugation with fluorescein isothiocyanate and its further purification(6). Subcutaneous areas of growing tendon adjacent to the knee joint of 8-10- and 14-16-day-old chicken embryos and of newborn, young (20 days), and adult chicken (3 months) were studied for development of normal tendon connective tissue. To examine the induced granulation tissue in adult chickens, the tendon of the knee joint was cut transversely under aseptic conditions, the skin was closed and the leg immobilized. The newly formed tissue was obtained from each animal at 4, 8, 14, 20 and 35 days. Tissue blocks from the growing tendinous areas were rapidly frozen and cut at 4 to 8 μ in the cryostat at $-20°C$, whereas other small pieces fixed in 4% formol or Carnoy fluid were stained with H & E, silver impregnation (Gridley method), toluidine blue (pH 5), and periodic acid Schiff (PAS) techniques for mucopolysaccharides and collagen fibers. Direct or indirect Coon's methods were applied to the unfixed or 95% alcohol or acetone treated frozen sections, using the labeled globulin fraction of the immune sera or the intact immune sera, followed by the fluoresceinated antirabbit globulin antiserum prepared in sheep. As control reactions in both

* Postdoctoral Fellow of Consejo Nacional de Investigaciones Cientificas y Téchnicas, Bueno Aires, Arg.

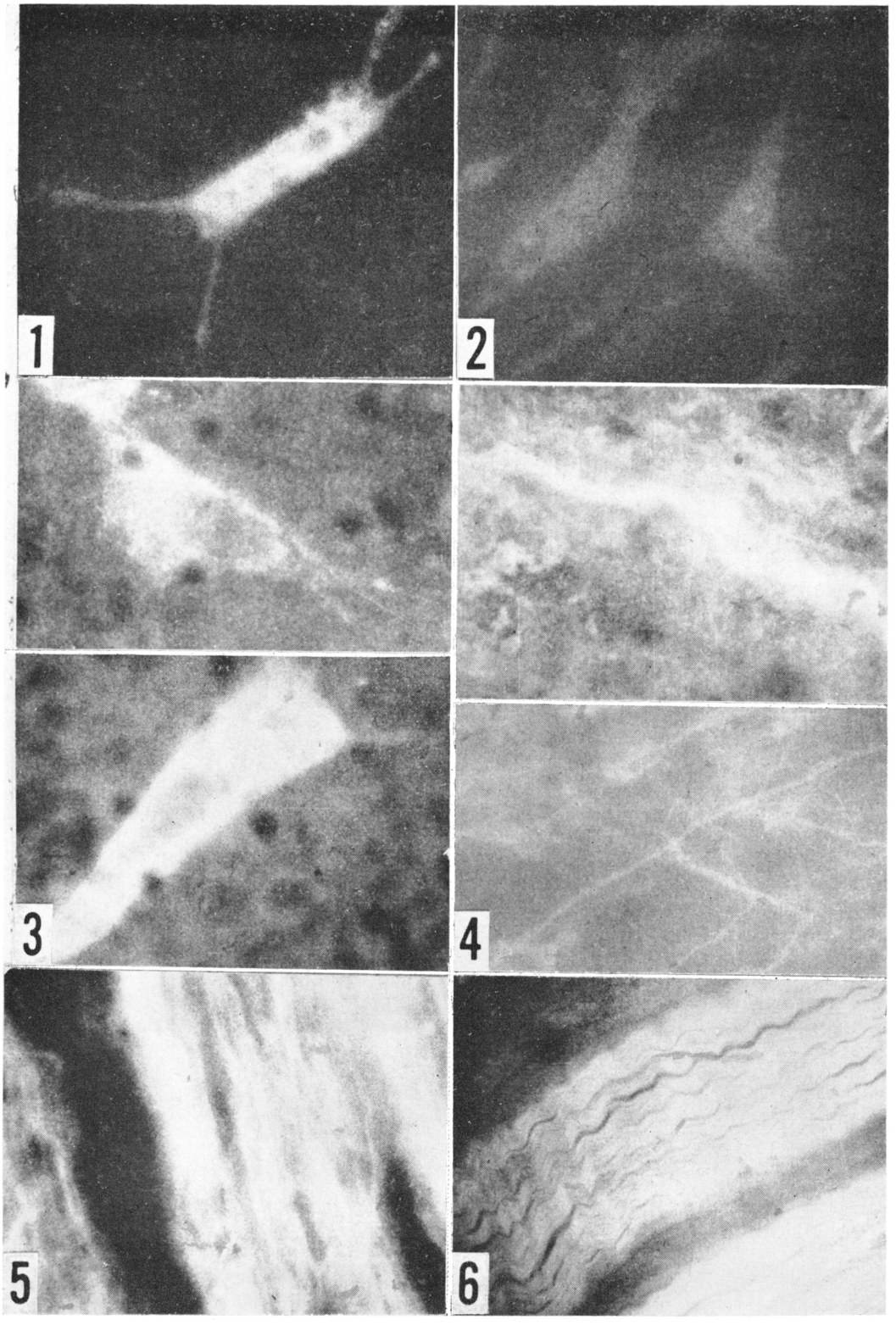
instances served: 1) sera or their globulin fraction from normal adult rabbits, 2) other immune sera or their globulin fraction such as rabbit sera antichicken testis, 3) unconjugated anticollagen sera of globulin fraction for blocking tests, 4) immune sera previously adsorbed *in vitro* in the presence of an excess of the antigen (10 mg of soluble or insoluble collagen fractions per ml of serum). In additional controls, soluble collagen was extracted from frozen sections with 0.14 M NaCl for 40 to 120 minutes before immunofluorescence study. The results with the extraction procedure were not rewarding since it altered the distribution of the specific fluorescence and often enhanced the nonspecific staining.

The proline and hydroxyproline ratios of the soluble and insoluble collagen fractions studied agreed with previous values for similar fractions(5). Fresh frozen sections were better than those previously fixed with 95% alcohol or acetone. However, fixation with acetone after staining improved the images and structures as compared with the direct mounting in glycerine. Treatment of conjugated globulin with Sephadex raised the intensity of the specific fluorescence and depressed nonspecific background staining more than purification with liver powder.

Results. In the 8- to 10-day-old embryos, the sites of dermal tissue where the tendon develops were undifferentiated connective tissue. Young fibroblasts, vessels and argyrophilic and PAS positive reticulin fibers were abundant. In the interfibrillar spaces amorphous mucopolysaccharides were visible. In this earlier stage soluble collagen antigen was demonstrated in the perinuclear region or diffuse in the cytoplasm of the young fibroblasts (Fig. 1 and 2), whereas insoluble collagen fluorescence was absent. Extracellularly, neither collagen antibodies were seen though polysaccharides and reticulin fibers had already developed (Fig. 3). At 14 to 16 days, the deep layer of the dermis was well delimited as site of the anterior tendon of the neighboring joint. Mature fibroblasts were present, reticulin fibers persisted, but newly and well oriented collagen fibers and bundles had formed. These fibers appeared more weakly PAS positive, and less argyrophilic than reticulin. During this period the immunofluorescent reaction persisted in a few cells and appeared over the collagen fibers (Fig. 4), whereas insoluble collagen was demonstrated only in collagen bundles (Fig. 5). In the newborn animal the tendon had mature fibroblasts and closely packed collagen bundles, as in the adult chicken. The fibroblasts gave no reaction and specific fluorescence for both soluble and insoluble collagen was detected in the intercellular fibers, and it paralleled the increase in the number and diameter of the collagen fibers, particularly in the well-packed bundles of final tendon (Fig. 6).

The repair process in the cut tendon went through stages, reflecting a progressive maturation of the granulation tissue between the tendon ends. Within 4 days a net of initially precipitated fibrin was invaded by newly formed vessels and young proliferating fibroblasts, while PAS positive and argyrophilic reticulin fibers appeared around the fibroblasts, as did scattered macrophages, plasmacytes and mast cells. At 8 days, young fibroblasts and reticulin fibers were abundant. During these two periods, most of the cells with features of fibroblasts contained soluble collagen. Fibroblasts matured, pale PAS stained and poor argyrophilic collagen fibers gradually developed and vascularization decreased from the 14th day on. The specific fluorescence in the cells disappeared, but the reticulin fibers or the vessel wall were not stained. Finally, from the 20th day to the 33rd day, mature fibroblasts were almost the only cell type present, whereas PAS negative, less argyrophilic but thicker collagen fibers and bundles replaced the reticulin fibers and interdigitated with the hyalinized ends of the cut tendon. The fibroblasts were negative but the collagen fibers and bundles positive, with both anticollagen antibodies. These reactions were weaker or absent in the hyalinized part of the injured tendon.

Other cells never reacted for collagen. Also (6), the neighboring tissues, the dermal skin of young and adult chicken, and the intact tendon near the injured area reacted well for both the soluble and insoluble collagen fractions.


1
2
3
4
5
6

Discussion. The histoimmunological demonstration of soluble and insoluble collagen fractions during the development of connective tissue is supported in the present study by better immunological techniques, *i.e.*, by purification and adequate proline-hydroxyproline ratio of the antigens, acceptable titers and specificity as shown by lack of cross reactions between soluble and insoluble collagen antibodies(5). The immunological specificity of the soluble and insoluble collagen fractions is confirmed by their specific localization in tissue structures. The soluble collagen fraction is detected inside or outside the developing fibroblasts, whereas the insoluble fraction only in the intercellular spaces.

During collagen synthesis, the neutral salt soluble fraction which is most easily extractable, is a precursor of both the acid soluble and insoluble fractions(8). Our study supports this concept because of the sequential appearance of these fractions in growing tissue. The localization is similar in the developing normal tendon and in granulation tissue of injured tendon. Neutral salt soluble collagen is first observed inside the young fibroblasts in active fibrillogenesis, to later disappear in the mature cells. Subsequently, the soluble fraction is extracellular when collagen fibers develop and the insoluble fraction appears in collagen bundles. The delayed reaction of insoluble collagen might be explained by the lower titer of the respective antibody(5) which requires a higher concentration of the antigen per unit area.

Specific anticollagen antibodies react only with antigens in collagen fibers and not with reticulin, elastic fibers, interfibrillar mucopolysaccharides or other connective tissue proteins. The absence of reaction between anticollagen antibodies and reticulin fibers previously reported(6) was confirmed. The difference in antigenic nature between collagen and reticulin fibers is suggested by the following: 1) when reticulin fibers first appear, no soluble collagen antigen is detectable outside the cells with the present immunofluorescent method used, and 2) the soluble extracellular collagen coincides with the appearance of collagen fibers. Also histochemically these two kinds of fibers differ by the PAS positivity and argyrophilia of the reticulin fibrils. With few exceptions(9), immunofluorescent observations with antibodies against rat glomeruli, lung or antisynovium extracts(10,11,12) presumably containing antireticulin antibodies, support this difference. Fluorescence in reticulin fibers of many organs and basement membranes does not coincide with collagen fibers.

Since the aminoacid composition of collagen and reticulin seems to be the same(13), the immunofluorescent demonstration of soluble collagen in cells suggests the synthesis of a common basic precursor for both collagen and reticulin. The absence of histoimmunological reaction of the early extracellular reticulin fibers which precede the collagen might result from some change in the antigenic structure of reticulin. This might be due to carbohydrates and lipids(14) in reticulin.

FIG. 1. Section of growing tendon (8-10-day-old chick embryo) showing fibroblasts and intercellular substance. Indirect Coon's technique using rabbit serum antichicken soluble collagen followed by sheep globulin antirabbit globulin. Fluorescence is seen in fibroblasts but not in intercellular areas. × 280.

FIG. 2. Same tissue section as in Fig. 1, incubated with normal rabbit serum as control. Indirect Coon's technique. Slight spontaneous fluorescence is observed inside fibroblasts. × 280.

FIG. 3. Same tissue section and technique as in Fig. 1 at higher magnification. Fluorescence appears in cytoplasm and in thin branches of fibroblasts. Negative reaction in intercellular spaces where reticulin fibers are already present. × 800.

FIG. 4. Growing tendon (14-16-day-old chick embryo). Section incubated with antisoluble collagen antiserum. Indirect Coon's technique. Slight fluorescence is present in first thin collagen fibers (bottom) but bright fluorescence appears over thicker collagen fibers (top). × 400.

FIG. 5. Same tissue section as that of Fig. 4. Incubation with antiinsoluble collagen antiserum. Indirect Coon's technique. Bright fluorescence is observed in fibers of developing collagen bundles. × 400.

FIG. 6. Tendon of newborn chicken. Incubation with antiinsoluble collagen antiserum. Indirect Coon's technique. Fluorescence is clearly seen in the well-packed collagen bundle. × 100.

Summary. The localization of soluble and insoluble collagen fractions during the development of normal chick embryo tendon and granulation tissue in injured adult chick tendon was studied by the immunohistochemical technique. Antisoluble collagen antibody reacted only with the cytoplasm of young fibroblasts and with extracellular young collagen fibers. Reticulin fibers which precede the collagen and interfibrillar mucopolysaccharides did not react nor did other tissue structures. Antiinsoluble collagen antibody did not react with fibroblasts and reticulin fibers but did react with collagen bundles.

1. Stearns, M. L., Am. J. Anat., 1940, v67, 55.
2. Porter, K., Biophys. J., 1964, v4, 167.
3. Heller, P., Jakulis, V. J., Zimmerman, H. J., Proc. Soc. Exp. Biol. and Med., 1959, v101, 509.
4. Watson, R. F., Rothbard, S., Vanamee, P. J., J. Exp. Med., 1954, v99, 535.
5. Paz, M. A., Davidson, O., Gomez, C., Mancini, R. E., Proc. Soc. Exp. Biol. and Med., 1963, v113, 98.
6. Mancini, R. E., Paz, M. A., Vilar, O., Davidson, O. W., Barquet, J., ibid., 1965, v118, 346.
7. Jackson, D. S., Bentley, J. P., J. Biophys. Biochem. Cytol., 1960, v7, 37.
8. Jackson, D. S., Biochem. J., 1957, v65, 277.
9. Rothbard, S., Watson, R. F., J. Exp. Med., 1962, v116, 337.
10. Cruickshank, B., Hill, A. G. S., J. Path. Bact., 1953, v66, 283.
11. Taylor, H. E., Shepherd, W. E., Robertson, C. E., Am. J. Path., 1961, v38, 39.
12. Scott, D. G., Immunology, 1960, v3, 226.
13. Berrens, L., Van Driel, L. M., Naturwiss., 1962, v49, 608.
14. Snelman, O., Acta Chem. Scandinav., 1963, v7, 1049.

Received February 23, 1965. P.S.E.B.M., 1965, v119.

Febrile Responses to the Intracisternal Injection of Endogenous (Leucocytic) Pyrogen in the Rabbit.* (30264)

RONALD D. ADLER AND ROBERT J. T. JOY
Department of Nutrition, Harvard School of Public Health, Boston, Mass.

Since the demonstration that fever following intravenous injection of a variety of bacterial agents and viruses is accompanied by the release of an endogenous pyrogen (EP) from host tissues, primarily leucocytes(2,9), attention has been directed to the site of action of this pyrogen and its role in the pathogenesis of fever(1). A current hypothesis suggests that EP produces fever by a direct action of the central nervous system (CNS) thermoregulatory centers(1). This hypothesis is supported indirectly by observations which contrast the effects of intracarotid and intravenous routes of administration(8), but it has not been tested by direct application of EP to the central nervous system(3). In this study endogenous (leucocytic) pyrogen prepared from rabbit leucocytes *in vitro* was applied directly to the CNS by intracisternal injection and the resulting fevers compared to those resulting from the same dose administered intravenously in the same rabbits.

Methods. Female white rabbits (2.6-4.0 kg) served as leucocyte donors and pyrogen recipients. Endogenous (leucocytic) pyrogen (LP) was prepared in a standard fashion(4) from polymorphonuclear leucocytes obtained from sterile saline-induced peritoneal exudates. Two stock solutions of LP were prepared from 2 different peritoneal exudates in such a way that each ml of LP solution contained the pyrogen from approximately 80 $\times$ 10^6 leucocytes. The standard dose was 0.1 ml of the stock solution. This was given undiluted in the intracisternal (i.c.) injections. and diluted to 1.0 ml with pyrogen-free 0.9% saline (Abbott) for intravenous (i.v.) injections.

* This work was supported in part by a grant-in-aid from Nat. Inst. of Neurolog. Dis. and Blindness (NB-01941 to Dr. Jean Mayer), N.I.H., U.S.P.H.S.; The Fund for Research and Teaching, Dept. of Nutrition, Harvard Univ. School of Public Health; and the Dept. of Biology, Harvard Univ.