PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **183**, 214–220 (1986)

Para Osteo Arthropathy—Ectopic Ossification of Healing Tendon about the Rodent Ankle Joint: Histologic and Type V Collagen Changes[1] (42407)

HENRY BROWN,[2] H. PAUL EHRLICH, PAUL M. NEWBERNE, AND TAKASHI KIYOIZUMI[3]

Department of Nutrition and Applied Food Science, Massachusetts Institute of Technology, Cambridge 02137; Shriners' Burn Institute, Boston, Massachusetts 02114; The New England Deaconess Hospital, Boston, 02215; The Veterans Administration Medical Center, Manchester, New Hampshire 03104; and the Departments of Pathology and Surgery, Harvard Medical School, Boston, Massachusetts 02115

Abstract. Ectopic bone formation, or para osteo arthropathy (POA), is commonly seen in humans following lesions to the central nervous system. The condition also occurs after severe trauma, burns, or tetanus, and, rarely, after poliomyelitis or cauda equina lesions. The lack of a suitable laboratory animal model has hampered study of its etiology and treatment. The lesion occurs just above the rodent ankle joint 7 weeks after repair of the severed achilles tendon. Tendon repair using a single silk suture resulted in ectopic calcification in 22 of 23 animals. Ectopic ossification, with extensive mineralization, occurred in 11 animals, 5 of which showed bone marrow. Ectopic mineralization was moderate in 8 others, minimal in 4, and absent in one. Collagen typing thrice during healing indicated that type V collagen increased by one-third at 10 days, doubled at 20 days, and returned to near normal amounts at 50 days. Since type V collagen is a major component of the nonfibrous collagen of blood vessels, increased amounts of type V collagen are consistent with the presence of many new blood vessels in the granulation tissue at 10 and 20 days. Vascularity is less dense at 50 days when ectopic mineralization and new bone formation are observed. These findings suggest that POA and the degree of maturation of healing tendon may be linked to reduced amounts of type V collagen. It is concluded that the sutured tendon of the rodent ankle joint offers an animal model by which to study ectopic bone formation, or para osteo arthropathy.

Para osteo arthropathy (POA) refers to ectopic ossification in and about muscular and fascial tissues of joints in paraplegic patients (1). Changes do not occur in the bones themselves. In the original descriptions, war injuries to the spinal cord were the most frequent causes, but POA also occurred in syphilitic and tuberculous myelitis. Rossier and others subsequently observed that the condition occurs in many upper motor neuron lesions and diseases, and after burns, trauma, tetanus, carbon monoxide poisoning, and the Guillian–Barre syndrome (2). It occurs rarely after poliomyelitis or lesions of the cauda equina. Dejerine's studies reported that the knee was the most commonly involved joint (1). Rossier found that ossification was more common in the hip and pelvis (2). In one report, using X-ray criteria, more than 40% of patients with total hip replacement developed opacities which might be interpreted as ectopic ossification (3). The ossified lesion of POA is new bone formation by true tissue metaplasia, as opposed to ectopic calcification. Its onset is marked by the classic signs of inflammation. Ossification occurs rapidly, often in a few days, and is generally irreversible. The cause of POA is unknown, although vascular anomalies have been suggested (2, 4). Study of the condition has been hampered by the lack of an animal model.

This paper reports POA in rodent achilles tendon after severing and immediate suture repair near the ankle joint. Changes in the

[1] Supported in part by grants from the American Society for Surgery of the Hand and from the Charles and Evelyn Marran Foundation, the Shriners of North America, and by USPH Grants GM 217000 and GM 25561. The authors thank Kurt C. Lange for his assistance with collagen analyses.

[2] To whom correspondence and reprint requests should be addressed at Box 26, 185 Pilgrim Rd., Boston, Mass. 02215.

[3] Present address: Department of Plastic and Reconstructive Surgery, Keio University Hospital, 35 Shinanomachi Shinjuku-Ku, Tokyo 160, Japan.

0037-9727/86 $1.50

amounts of type V collagen in healing tendons at three points during healing, including times when tissues were calcifying and forming new bone, are presented.

Materials and Methods. POA, characterized by metaplasia and ectopic ossification, occurred experimentally approximately 50 days after repair of both rodent achilles tendons by the following technique.

Fifty-one 120-g male and female Sprague–Dawley rats (Charles River Laboratories, Wilmington, Mass.) were individually ether anesthetized. A 5-mm incision was made proximal to the achilles tendon insertion at the heel. Care was taken to keep the incision superficial so as not to cut the tendon. The tendon was isolated by blunt dissection from underlying tissue with a small curved forceps which was left spread beneath the tendon to prominently expose it. A 4-O silk suture was passed through the tendon as far proximally as possible and then as far distally as possible near its heel insertion to form a loop (Fig. 1). The tendon was transected between the spread forceps in the middle of the loop without cutting the loop. Both tendon ends, when cut, retracted out of the field into the muscle mass. They were brought together by pulling on the suture. The cut ends were reapproximated by tying the suture and the incision was closed. Animals were placed on soft fiber or plastic chips in individual cages where they were allowed a standard chow diet and water *ad libitum.* They were killed quickly 10, 20, and 50 days after repair by being placed in an atmosphere of carbon dioxide.

A repaired achilles tendon from one limb of each of 4 animals 10 days after repair, 4 animals at 20 days, and 23 animals at 50 days was fixed in 10% Formalin. They were sectioned and stained with hematoxylin and eosin. Longitudinal sections of the entire tendon (about 1 cm) were examined under light microscopy and photographed with Kodak Panatomic X film.

Repaired tendons from the opposite limbs were placed in 0.5 *M* acetic acid and stored until use. Four tendons from each group were combined to obtain sufficient material for each collagen analysis. Since only one analysis could be made of the 10- and 20-day groups, eight additional animals were prepared for study at 10 days and eight at 20 days after repair to confirm the first analytical findings. Sufficient 50-day tendons were available for confirming studies.

Collagens were extracted by limited pepsin digestion at 4°C in 0.5 *M* acetic acid and stirred for 48 hr using 100 μg pepsin (Boehringer Mannheim, Indianapolis, Ind.) per 5 mg tissue wet weight. The digest was centrifuged for 15 min at 10,000*g*. The supernate containing soluble collagens was saved. The insoluble pellet was further digested for 48 hr in the same manner. The supernates were combined after centrifugation. Pepsin was inacti-

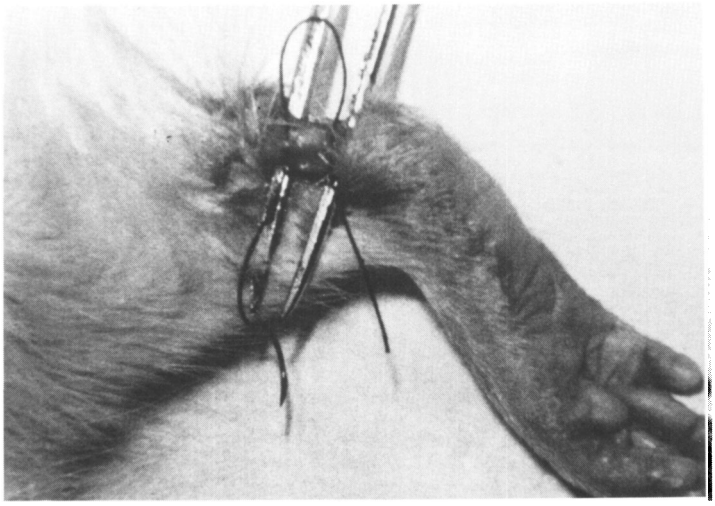

FIG. 1. Normal rodent achilles tendon with suture loop in place.

vated by adjusting the pH to 8 using sodium hydroxide. Sodium chloride was added to a final concentration of 20% w/v to precipitate collagens. This mixture was stirred overnight at 4°C and the precipitated collagen was collected by centrifugation at 15,000*g* for 15 min. Bulk salt fractionation of collagens was carried out in the following manner. The precipitate was suspended in 2.5 ml of 2.2 *M* NaCl in 145 m*M* potassium phosphate buffer, pH 7.6. After stirring and centrifuging as above, the supernate was saved and labeled "2.2 *M* NaCl fraction." The insoluble residue was suspended in 2.5 ml 1 *M* sodium chloride, buffered as before, stirred, centrifuged, and its supernate labeled "1 *M* NaCl fraction." The insoluble material was then resuspended in 2.5 ml potassium phosphate buffer alone, stirred, centrifuged, and the supernate was labeled "PO_4 soluble fraction." The insoluble residue was discarded.

Further characterization of collagen types was made by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The system used 0.1% SDS buffered at pH 9 with Tris–sulfate and an Ortec pulsed power supply (Oak Ridge, Tenn.) (5). Analyses were made of 125-μg samples, each in a 25-μl vol. Gels were stained with 0.5% Coomassie brilliant blue R-250.

Type V collagen is soluble in the "2.2 *M* NaCl fraction." Type I collagen is the major one present in the "1 *M* NaCl fraction" and type III collagen is the major one present in the "PO_4 soluble fraction." The α 1(V) polypeptide of type V collagen (6, 7) was measured by quantitative densitometry in these experiments. Its identification by electrophoresis has been previously confirmed in this laboratory (8, 9).

Results. All animals walked immediately after surgery and no obvious impairment of their mobility appeared during healing. One consequence of healing was the increased diameter of the tendon to several times the original. Dull, thick, gray scar tissue replaced the glistening, smooth white tendon in the sutured area. At 50 days, some scar felt gritty when cut, and flecks of mineralized tissue were grossly visible.

Histological studies showed normal achilles tendon (Fig. 2A) to be composed of tightly packed parallel collagen fibers ("C") with relatively few cells (black circle). Cartilage and bone cells (open white squares) are visible distal to the tendon attachment (open white arrow) of the os calcis.

Granulation tissue (Fig. 2B) with large blood vessels ("L") and small blood vessels ("S"), a few giant cells (circle, "G"), fatty areolar tissue ("A"), and an occasional nerve (above "N") is present at 10 days. At 20 days (Fig. 2C) granulation tissue containing many blood vessels ("S") and a few giant cells (arrows) is also prominent. Small round cells in clumps are seen at both 10 and 20 days ("R"). Polymorphonuclear leukocytes interspersed with round cells are seen at higher magnifications also at 10 and 20 days, but their nuclei are not clearly seen in the photographs.

Metaplasia and mineralization occurred after 50 days in 22 of 23 animals. Metaplastic changes are classified as minimal, moderate, or extensive. Extensive metaplasia is indicated when more than half of three low power fields have a cartilaginous appearance with cartilage-like cells and fibroplasia. Moderate metaplasia is characterized by less than half of three low power fields having cartilage-like cells. An occasional cartilage-like cell in three low power fields is interpreted as minimal metaplasia. Homogeneous dark staining around cartilage-like cells is characteristic of ectopic calcification (Fig. 2D), while woven bone spicules denote actual new bone formation (Fig. 2E). Areas of cartilage and calcified new bone are readily seen at 50 days. Cartilaginous metaplasia with cartilage cells blending into mineralized tissue is confirmed by special cartilage and bone stains, periodic acid–Schiff (PAS), and Alcian blue (10, 11). Five specimens show developed marrow, confirming that ossified areas are bone (Fig. 2E). Little granulation tissue is evident, and the density of cells of the repaired area, the scar, is greatly reduced compared to that seen at 20 days. Giant cells, indistinguishable from osteoclasts, appear in the ossified area at locations consistent with bone resorption (Fig. 2E). A chronic inflammation persists, marked by round cells and giant cells in scar tissue (Fig. 2C, E).

The electrophoretic pattern of collagen types from rat achilles tendon at different stages of healing is shown in Fig. 3. The α 1(V) polypeptide is indicated in the margin. This band is most prominent in the "2.2 *M* NaCl

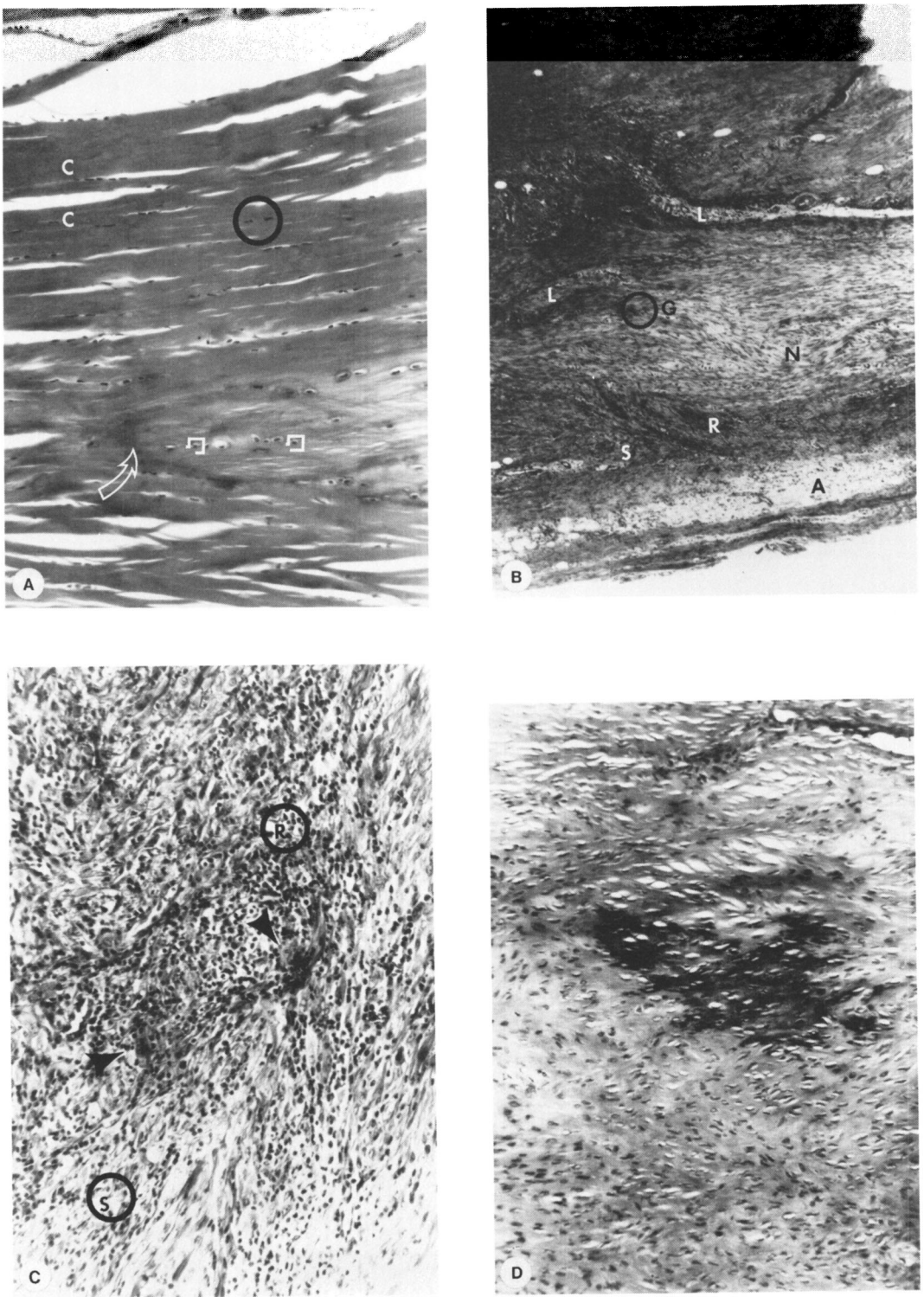

FIG. 2. (A) Normal achilles tendon at the level of the os calcis. H and E stain. Original magnification ×60 in A and B. (See text for explanation of arrows, letters, and symbols on A–C.) (B) Cellularity of healing tendon at 10 days. (C) Inflammatory response with giant cells (arrows) 20 days after repair. Original magnification ×150 in C–E. (D) Ectopic calcification of new cartilage seen in the dark staining area of the upper center 50 days after tendon repair. (E) Woven bone spicules with marrow and osteoclasts (arrows) 50 days after repair.

FIG. 2—*Continued.*

fraction" lanes labeled "1" for each time point. Visual comparison of "2.2 *M* NaCl fraction" shows the intensity of the α 1(V) band 10 days after tendon severance greater than controls, even greater at 20 days, and nearer normal after 50 days. This experiment was repeated twice with similar results. Densitometry tracings of amounts of α 1(V) polypeptide from one such experiment are shown in Fig. 4. Measurement of areas under tracings indicate that the amount of α 1(V) polypeptide relative to total collagen in the fraction increased about one-third at 10 days (36%), more than doubled at 20 days (120%), and returned to close to control amounts at 50 days. This band is in the position originally described by Burgeson *et al.* (6).

Discussion. This model resembles clinical POA (1, 2) in the following ways: (i) New bone, rather than ectopic tendon calcification, is formed; (ii) bone forms near a joint, i.e., the ankle; (iii) an inflammatory response occurs; (iv) connective tissue is ossified; (v) new bone formed at multiple sites has features of spongiosal or woven bone, yet it is different from normal bone in adjacent areas; (vi) the lesion is associated with trauma.

Ectopic mineralization, called calciphylaxis, may occur by a different mechanism than POA. According to Selye, calciphylaxis demonstrates an endogenously regulated biologic mechanism which selectively induces calcium precipitation at certain sites and rarely continues on to true bone formation (12). Ectopic mineralization of turkey leg tendons has been described (10). The authors are not aware, however, of previously described ectopic bone formation in healing rodent tendon.

Healing tendon not only provides a laboratory model for studying POA, but its changes in type V collagen offer several comparisons. The increase in type V collagen is similar to one seen in several diverse tissues when they are compared to their normal or adult counterparts. Examples of these are hypertrophic scar (13), embryonic tissue (6), inflamed tissue (described here), and Dupuytren's contracture (8), all of which are characterized by increased vascularity (14). Type V collagen occurs in blood vessels (15). The high vascularity of those tissues may partially account for increased amounts of type V collagen. In each case, increased amounts of type V collagen are accompanied by immature connective tissue undergoing rapid turnover and remodeling (8). One may theorize that this circumstance

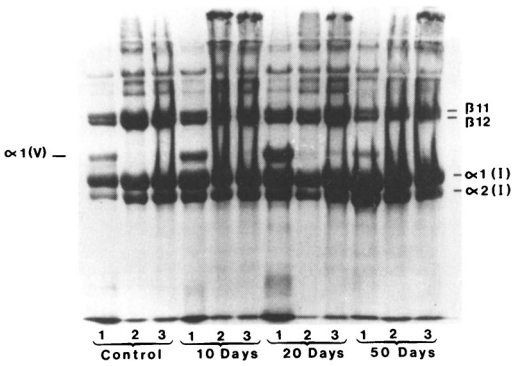

FIG. 3. Collagens stained on polyacrylamide gel after separation by electrophoresis. Polypeptide chains of different collagens are labeled in the margins. Numbers beneath the tracings indicate the fractions separating at different salt concentrations: (1) 2.2 *M* NaCl fraction; (2) 1 *M* NaCl fraction; (3) PO_4 soluble fraction.

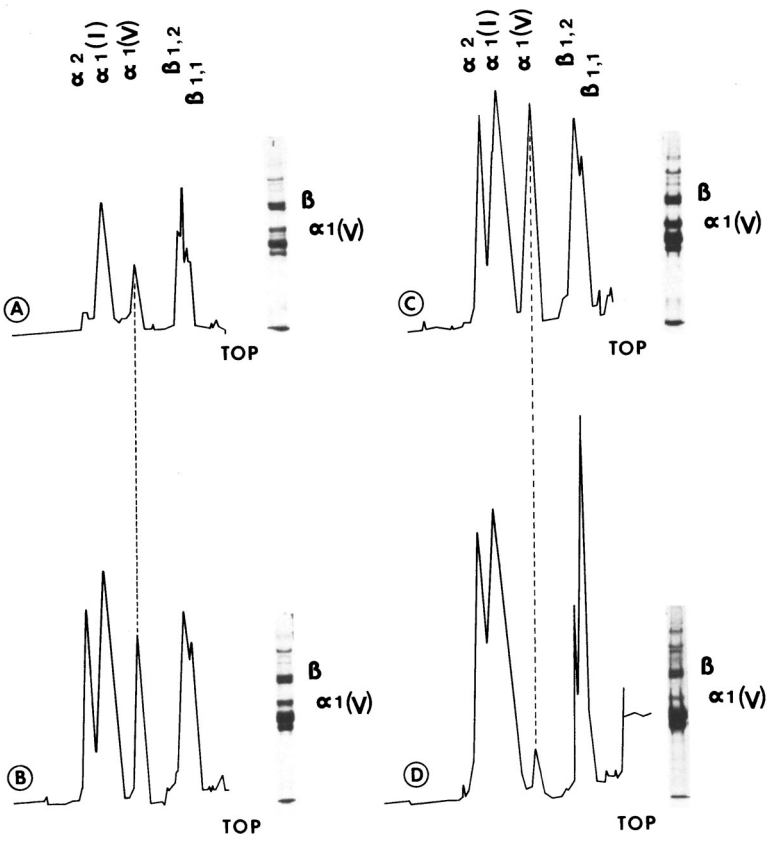

FIG. 4. Densitometry tracings show the amounts of α 1(V) polypeptide relative to amounts of other collagens in the 2.2 *M* NaCl fraction at different time intervals. (A) Control tendons; (B) 10 days after tendon repair; (C) 20 days after tendon repair; (D) 50 days after tendon repair.

may promote mineralization in POA and fibrotic contracture.

Type V collagen, which is found in the "2.2 *M* NaCl fraction," may, at times, also appear in the "1 *M* NaCl fraction." This unexplained aberration also occurred in fractionating Dupuytren's diseased palmar fascia (8). The reason for this unexpected finding is not evident.

The observation that amounts of type V collagen varied predictably at different times during tendon healing in these experiments suggests that it may be a chemical marker of the stages of tendon healing. Amounts of type V collagen might, therefore, be studied clinically in healing tendon as an aid to proper timing of surgical procedures, for example, the surgical release of tendon adhesions.

1. Dejerine M, Ceillier MA, Dejerine Y. Para-osteo-arthropathies des paraplegieques par lesion medullaire: Etude anatomique et histologique. Rev Neurol **5**:399–409, 1919.
2. Rossier AB, Bussat PH, Infante F, Zender R, Courvoisier B, Muheim G, Donath A, Vasey H, Taillard W, Lagier R, Gabiani G, Baud CA, Pouezat JA, Very JM, Gachen HJ. Current facts on para-osteo-arthropathy (POA). Paraplegia **2**:36–78, 1973.
3. Salvati EA, Wilson PD, Jolley MN, Vakili F, Aglietti P, Brown C. A ten year follow-up study of our first 100 consecutive Charnley total hip replacements. J Bone Joint Surg **63A**:753–767, 1981.
4. Galibert P, Lopez C, Cecile JP, *et al.* Etude angiographique de la circulation des membres inferieurs aux differents stades evolutifs d'une paraplegée. Neuro-Chir **7**:181–201, 1961.
5. Miner GD. In: Ortec Application Note AN-32A, Oak Ridge, Tenn., Ortec Pub., p39, 1973.
6. Burgeson RE, El Adli FA, Kaitila II, Hollister DW. Fetal membrane collagens: Identification of two new collagen chains. Proc Natl Acad Sci USA **78**:2579–2583, 1976.

7. Bornstein P, Sage H. Structurally distinct collagen types. Ann Rev Biochem **49**:957–1003, 1980.
8. Ehrlich HP, Brown H, White BS. Evidence for type V and type I trimer collagens in Dupuytrens contracture palmar fascia. Biochem Med **28**:273–284, 1982.
9. Trelstad RL, Lawley KA, Hayashi K, Ehrlich HP, Silver FH. Type V collagen from chick embryo: Biochemical, physicochemical and ultrastructural characteristics. Coll Res **1**:39–52, 1981.
10. Johnson LC. Mineralization of turkey leg tendon. 1. Histology and histochemistry of mineralization. In: Sognnaes RF, ed. Calcification in Biological Systems. Washington, D.C., Amer Assoc Adv Sci, Publication No. 64:pp119–123, 1964.
11. Smith HA, Jones TC, Hunt RD. Veterinary Pathology. Philadelphia, Lea & Febiger, p1069, 1972.
12. Selye H. Calciphylaxis. Chicago, Univ of Chicago Press, p460, 1962.
13. Ehrlich HP, White BS. The identification of A and B collagen chains in hypertrophic scar. Exp Mol Pathol **34**:1–8, 1981.
14. Ehrlich HP, Newberne PM, Brown H: Type V collagen and vascularity in wound healing. Surg Forum **36**: 531–533, 1985.
15. Madri JA, Furthmayr H. Isolation and tissue localization of type AB_2 collagen from lung parenchyma. Amer J Pathol **94**:323–330, 1979.

Received July 11, 1985. P.S.E.B.M. 1986, Vol. 183.
Accepted July 10, 1986.