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Morphological and Biochemical Effects of Sodium Morrhuate on Tendons

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Summary: The purpose of this study was to determine some of the morphological and biochemical effects of sodium morrhuate injections into intact rabbit patellar tendons and Achilles tendons. The effects of one, three, and five 100 μl injections of sodium morrhuate on tendon circumference, cell content, collagen fibril diameter, collagen-proteoglycan relationships, water content, amino sugar content, and hydroxyproline content were investigated over periods of 1, 4, and 9 weeks. In general, sodium morrhuate injected tendons were larger in diameter and contained more cells, smaller collagen fibrils, increased water and amino sugar content, and reduced hydroxyproline content compared with their contralateral controls. As a sclerosing agent, sodium morrhuate appears to mimic the early stages of an injury-repair sequence when injected directly into intact tendons. Whether sodium morrhuate may hasten repair responses or improve joint laxity remains to be determined. Key Words: Sclerotherapy—Sodium morrhuate—Tendons—Collagen—Ligaments.

In 1983 Liu et al. (13) published a paper describing the influence of the sclerosing agent sodium morrhuate on the rabbit medial collateral ligament (MCL) junction strength and demonstrated that repeated injections of 100 μl of a 5% sodium morrhuate solution directly into the MCL significantly increased the bone-ligament-bone junction strength and the ligament mass and thickness compared with saline injected controls. However, the underlying mechanisms for these changes were not resolved. The present study was undertaken to gain insight into the morphological and biochemical aspects of patellar tendons (PT) and Achilles tendons (AT) and to see if any of these features were altered by a series of sodium morrhuate injections directly into the tissues.

MATERIALS AND METHODS

One PT and one AT of nine 6 pound New Zealand rabbits were injected once per week with 100 μl of 5% sodium morrhuate (Morrhuate Sodium injection, USP, Eli Lilly Co., Indianapolis, Indiana). The opposite limbs were injected with an equal volume of 3% ethanol adjusted to pH 10 to duplicate the alcohol content and pH of the sclerosing solution. All injections were placed at the midpoint of the PT and in the center of the AT 1½ cm proximal to the insertion of the tendon into the calcaneus. The animals were identified according to the number of injections received and the time elapsed between the last injection and the excision of the tissues, i.e., 1 + 1 indicates one injection and sacrifice 1 week later, 3 + 9 indicates three injections and sacrifice 9 weeks after the third injection. The number of injections and time periods were as follows: 1 + 1, 1 + 4, 1 + 9, 3 + 1, 3 + 4, 3 + 9, 5 + 1, 5 + 4, and 5 + 9. Therefore, the effects of one, three, and five injections over time periods of 1, 4, and 9 weeks were investigated. At sacrifice the entire PT and a 1 cm piece of the AT taken from a site 1 cm above the insertion of the tendon into the calcaneus were removed for analysis. Each specimen was split longitudinally into three equal-size strips. Each longitudinal strip was cut in cross sections to form

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three smaller equal-size pieces per longitudinal strip, thereby dividing the original structure into nine equal-size pieces. The proximal and distal pieces from each strip were discarded and the central three pieces used for analysis. Electron microscopic data were obtained from the central piece, biochemical data from the medial piece, and light microscopic data from the lateral piece. The parameters measured were (a) tendon circumference, (b) cell content, (c) collagen fibril diameter, (d) collagen-proteoglycan relationships, (e) water content, (f) amino sugar content, and (g) hydroxyproline content.

Circumference

Circumferences were measured *in situ* by wrapping a 3–0 suture around the center of the PT at a point 1 cm proximal to the insertion of the AT. The suture was removed and the suture length required to circumscribe the structure was measured to the nearest 0.5 mm.

Cell Content

Cell content was determined from electron micrographs in which the number of nuclei present was counted in five electron micrographs from each specimen taken at 2,000 magnification and converted to the number of nuclei per square millimeter.

Collagen Fibril Diameter

Collagen fibril diameters were determined from cross-sectional electron micrographs taken at 20,000 magnification. One hundred fibrils from each of five micrographs per specimen were measured using a Bioquant Image Analysis system connected to an Apple II-e microcomputer. Measures were recorded in nanometers. The mean values of 500 fibrils/specimen and the frequency distributions of the 500 fibrils were determined for each specimen.

Collagen-Proteoglycan Relationships

A portion of each specimen was excised and fixed in glutaraldehyde and ruthenium red as originally described by Luft (14) and later modified by Laros and Cooper (12). These specimens were cut longitudinally and electron micrographs taken at 20,000 and 50,000 magnification to determine the presence and location of proteoglycans in relation to collagen.

Water Content

Water content was determined by the difference in wet and dry weight of a small portion of each ligament and tendon. Wet weight was measured immediately on removal of the sample and dry weight determined after thorough drying in a vacuum oven at 100°C.

Amino Sugar Content

After determination of the water content, the dry tissue specimens were suspended in 2 ml 0.1 *M* phosphate buffer, pH 6.5, containing 10 μl/ml papain (Sigma, type III), 5 m*M* cysteine hydrochloride, 5 m*M* EDTA, and digested overnight at 60°C. The digests were clarified by centrifugation, extracted with 1 ml chloroform, and precipitated with 10% cetylpyridinium chloride (CPC). The glycosaminoglycan-CPC complexes were allowed to form at room temperature overnight, collected by centrifugation, dissolved in 4 N HCl, and hydrolyzed for 5 h at 110°C. The hydrolysates were dried, redissolved in 0.1 *M* HCl, and the amino sugars determined with an amino acid analyzer (JEOL-6AH).

Hydroxyproline Content

An aliquot of the papain digests, prepared as described above, was made 6 N in HCl and hydrolyzed at 130°C for 3 h. Hydroxyproline was determined by the method of Woessner (24).

RESULTS

Morphology

Grossly, the control tissues were firm and glistening white in appearance. No inflammatory response was evident due to the 3% ethanol injections. At the light microscopic level, the fiber bundles were densely packed with thin spindle-shaped fibrocytes separating the collagen fibers (Fig. 1A). There was no discernible difference between the PT and the AT.

The majority of the sodium morrhuate injected specimens differed markedly in appearance from the control specimens. Although there were no ap-

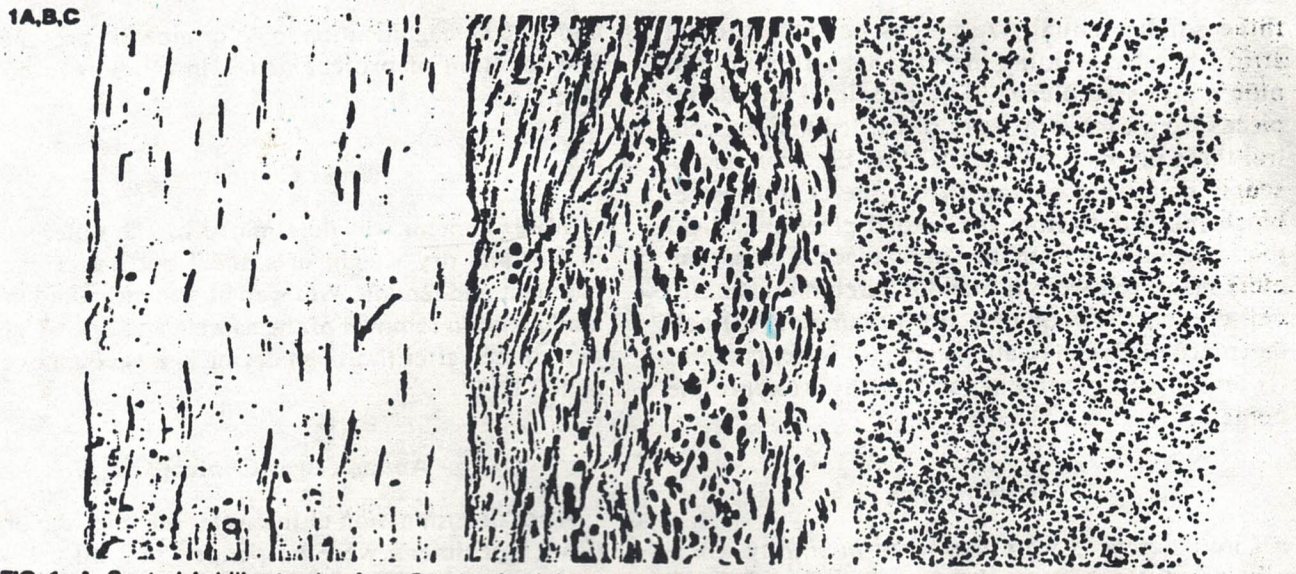

FIG. 1. A: Control Achilles tendon from 5 + 1 animal demonstrates dense longitudinal collagen fiber bundles and thin spindle-shaped fibrocytes (×160). B: Sodium morrhuate injected Achilles tendon from 5 + 1 animal demonstrates cellular hyperplasia and more loosely arranged collagen fibrils (×160). C: Low power micrograph of granulation tissue mass associated with sodium morrhuate injected 5 + 1 Achilles tendon (×100).

parent changes in the tissues receiving only one injection, those receiving three and five injections were yellowish in color. Microscopically there was an increase in cell content, in both number and kinds of cells present. This was maximized in the 3 + 1 AT and the 5 + 1 PT, which had large granulation tissue masses attached to their surface. Microscopically these consisted of neutrophils, lymphoctyes, plasma cells, and unidentifiable cell types with strands of collagen fibers intertwined among the cells (Figs. 1B and C). No specific orientation appeared to be present within the mass although the longitudinal axis of the mass paralleled the longitudinal axis of the tendons.

Circumference

The mean value of the injected PT was 19.2 ± 3.3 mm compared with 15.1 ± 2.0 mm for the controls. The same pattern existed for the AT in which the injected tendons averaged 16.7 ± 3.6 mm in circumference compared with 12.9 ± 2.4 mm for the controls. The mean values alone are not very representative for the injected limbs due to the variations in the number of injections and the time periods following the last injection. Therefore, a plot of the data presents a more meaningful comparison in which it is evident that the control tissues were quite uniform in size and always smaller than those of the injected limbs, with the one exception of the 1 + 4 PT in which both sides were equal in circumference (Fig. 2).

Cell Content

The number of cells per square millimeter of tissue was quite consistent in the control specimens but varied markedly in the injected tissues (Fig. 3). One injection of sodium morrhuate appeared to have little effect on the cell content with the possible exception of the 1 + 9 specimens, but those receiving three and five injections demonstrated a considerable increase in cell number. Further, there appeared to be a time factor involved in which cell numbers tended to decrease with time following the last injection. Aging did not appear to be a factor in the decreased number of cells since the oldest control specimens (3 + 9 and 5 + 9) did not differ to any extent from the younger control specimens. These results closely paralleled the water content of the tissues (Fig. 7).

Collagen Fibril Diameter

Frequency distributions indicated that one injection of sodium morrhuate appeared to have little effect on the collagen fibril diameters comprising the tendons with the possible exception of the 1 +

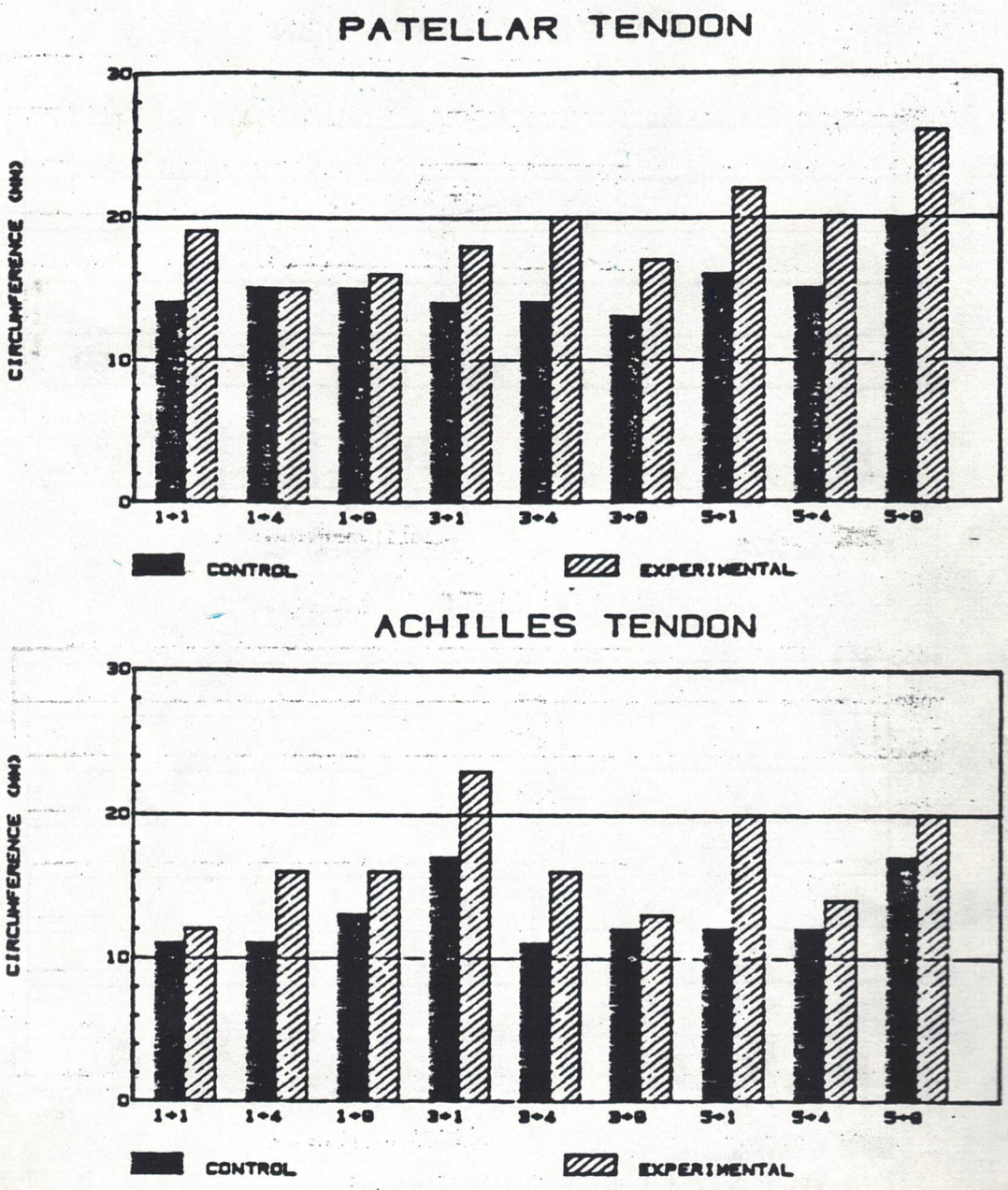

FIG. 2. Circumference of patellar tendons and Achilles tendons.

9 animals. However, in the three and five injection animals the control specimens exhibited flat or slightly unimodel curves, whereas the sodium morrhuate tissues revealed skewed distributions consisting of a large number of very small fibrils (Fig. 4). This was particularly evident in the animals receiving five injections. An example of this is illustrated in the electron micrographs (Fig. 5) used to plot the data presented for the 5 + 9 PT in Fig. 4. Some animals showed a marked up swing at 300 nm, but it should be noted the last category (300 nm) includes all fibrils greater than 280 nm in size.

Collagen-Proteoglycan Relationships

Ultrastructural localization of proteoglycans using ruthenium red (RR) did not show a consistent difference between control and experimental tissues; however, both the number and size of the RR particles were markedly increased in some of the sodium morrhuate injected tissues. Figure 6 illustrates one such example for the 5 + 1 AT. Similar results were obtained for the 3 + 1 specimens, and obvious although smaller differences were apparent for the 3 + 4 and 5 + 4 specimens. At 9

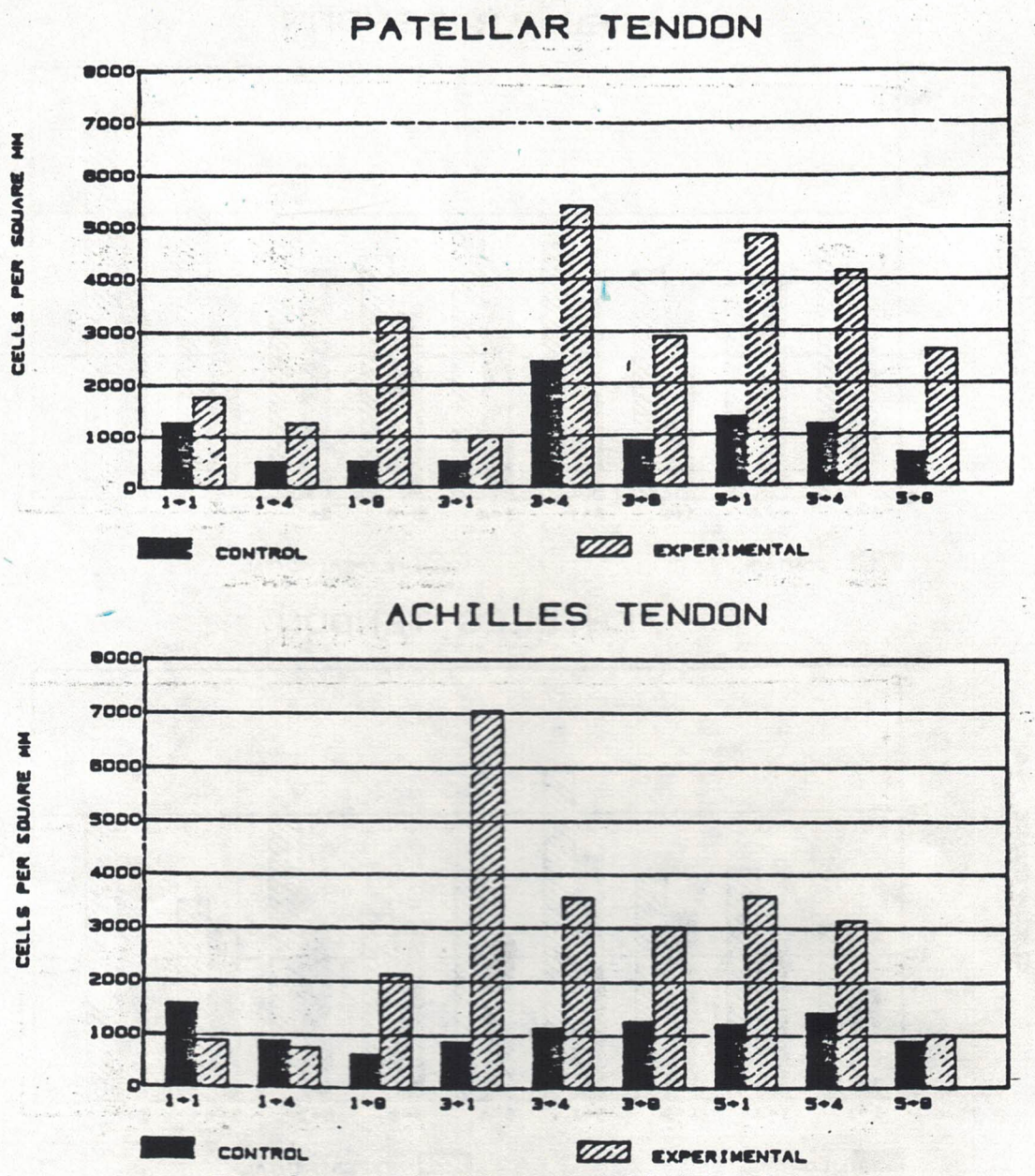

FIG. 3. Cell content in patellar tendons and Achilles tendons.

weeks there was no discernible difference in the concentration or size of the RR particles except that they were primarily on the small diameter fibrils in the experimental tendons. Animals receiving only one injection were indistinguishable from controls.

Water Content

Water content averaged 63% in the PT and 59% in the AT of control tissues. The average value for water content was not determined for the injected tendons due to the variation in the number of injections given; however, after three and five injections of sodium morrhuate, all specimens demonstrated an increase in water content that was particularly striking for the 3 + 4 and 5 + 4 PT and the 3 + 1, 3 + 4, 3 + 9, 5 + 1, and 5 + 9 AT specimens (Fig. 7). This change paralleled the increased cell content of the treated tissues (Fig. 3).

Amino Sugar Content

The total amino sugar content (glucosamine plus galactosamine) closely paralleled the changes in water and cell content of the tissues and tended to

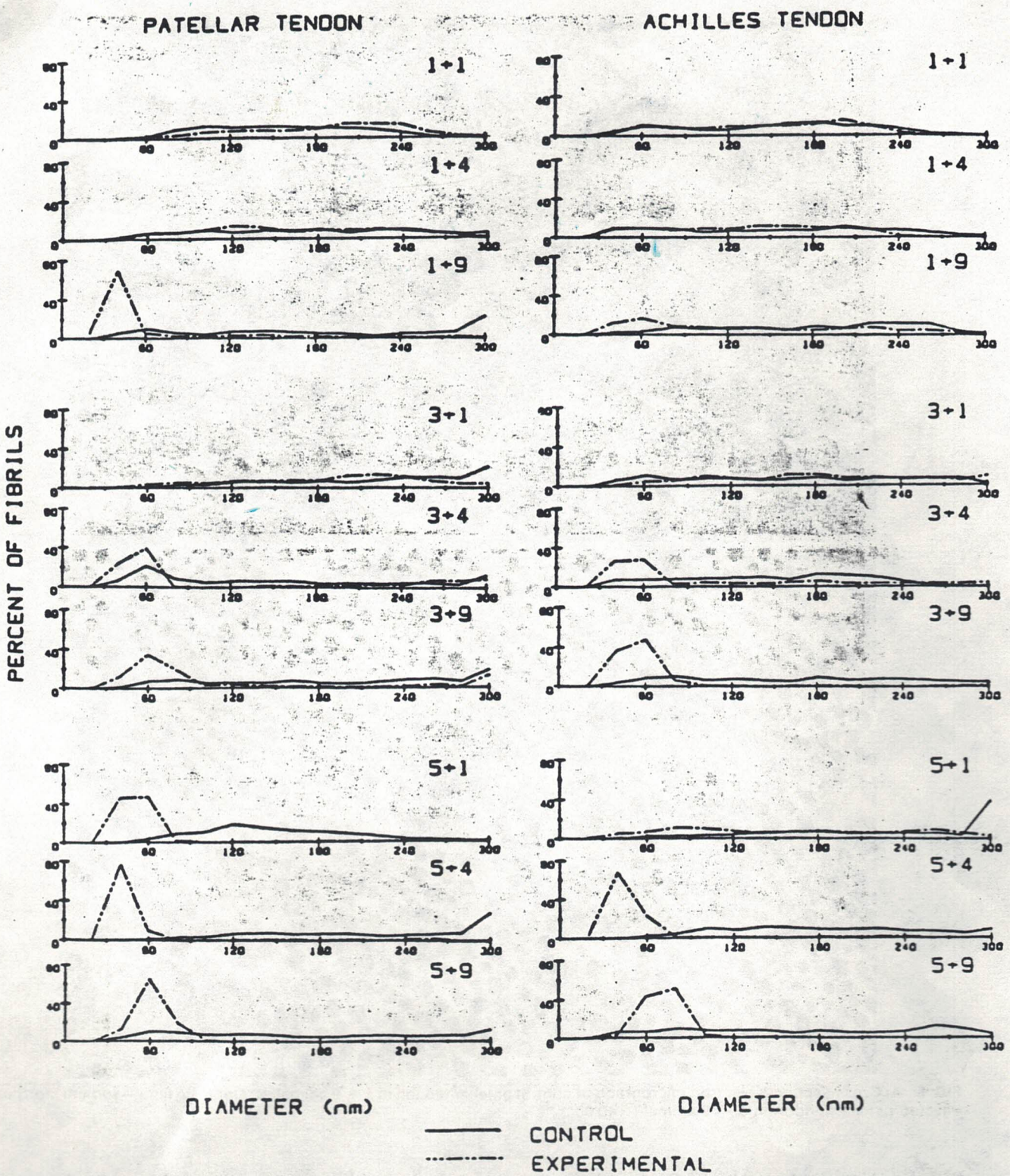

FIG. 4. Collagen fibril diameters in control and sodium morrhuate injected patellar and Achilles tendons.

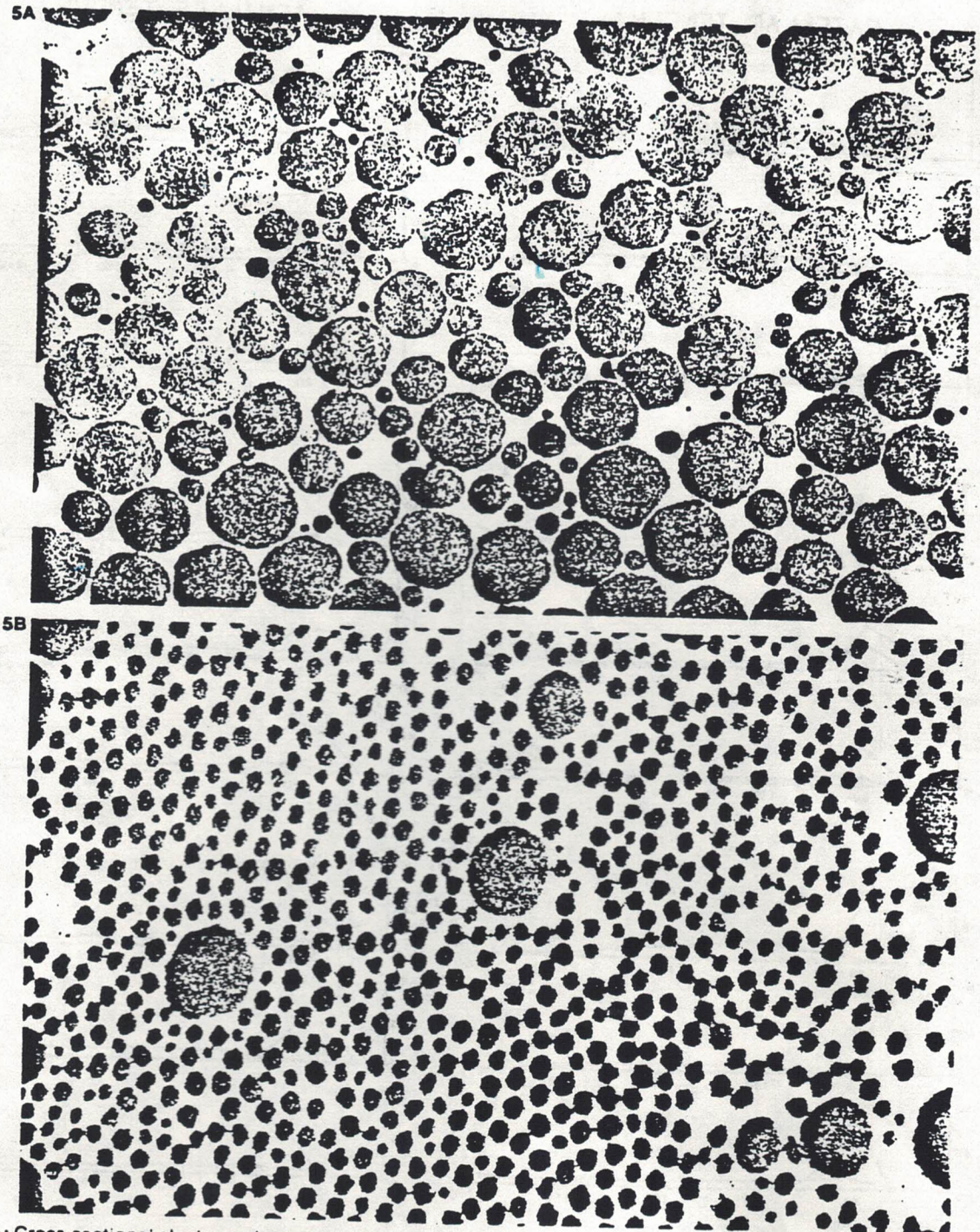

FIG. 5. A: Cross-sectional electron micrograph of control patellar tendon in 5 + 9 animal. B: Opposite limb—sodium morrhuate injected patellar tendon in 5 + 9 animal (×40,000).

be inversely related to the hydroxyproline content (Fig. 8). This was readily apparent for the 3 + 4, 3 + 9, 5 + 1, 5 + 4, and 5 + 9 specimens. The sodium morrhuate injected tissues consistently had higher amino sugar concentrations than the control specimens with the exception of the 1 + 4 PT and AT and the 3 + 1 PT, which were essentially equal to the control samples.

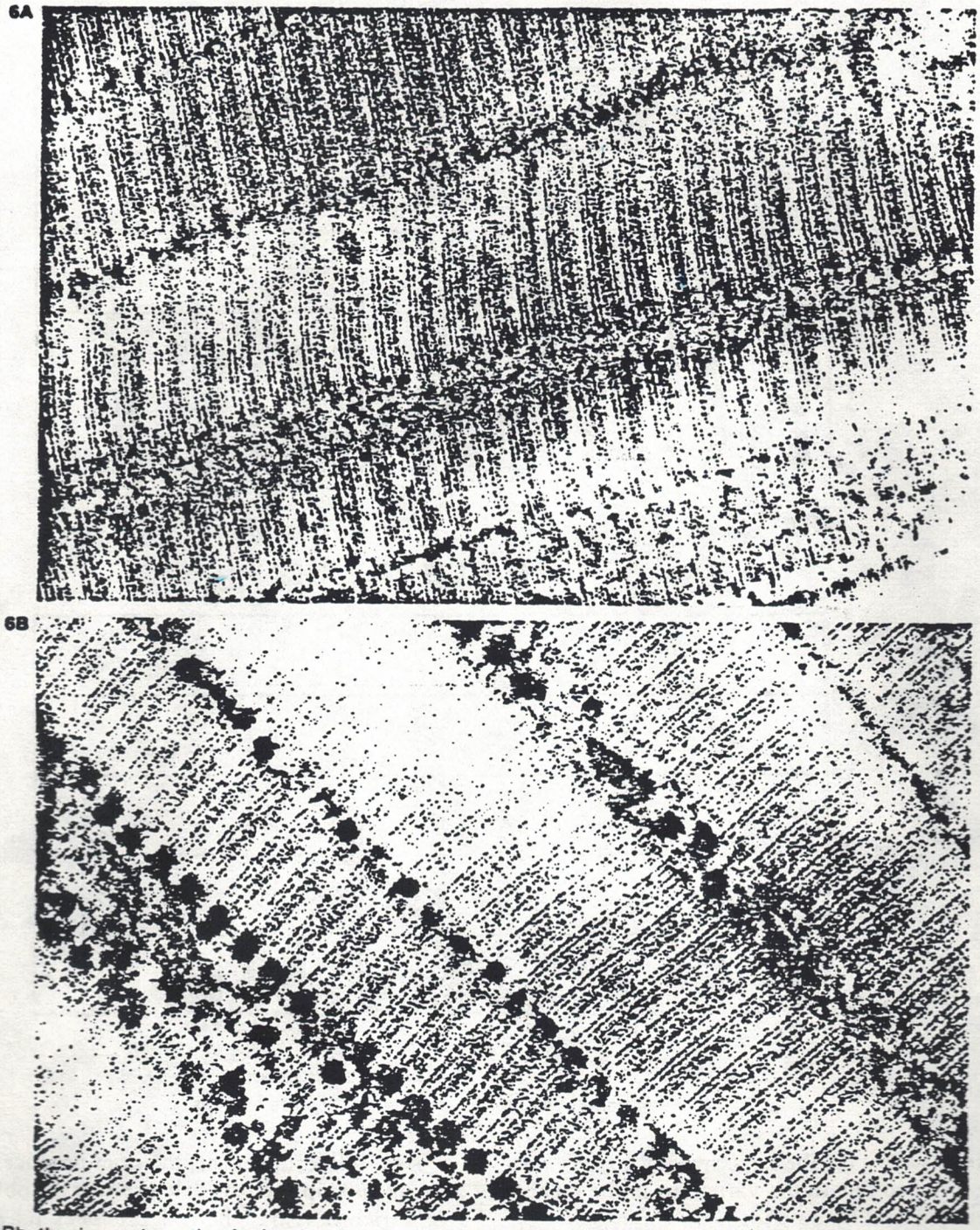

FIG. 6. Rhuthenium red reaction for localization of collagen-proteoglycan relationships. A: Control 5 + 1 Achilles tendon (AT). B: Sodium morrhuate injected 5 + 1 AT (×100,000).

Hydroxyproline Content

Hydroxyproline content was in general inversely related to amino sugar and water content in that most of the experimental tissues demonstrated lower hydroxyproline values (Fig. 9). Mean total collagen content (hydroxyproline × 7.14) was 82.2% in the control PT and 79.5% in the control

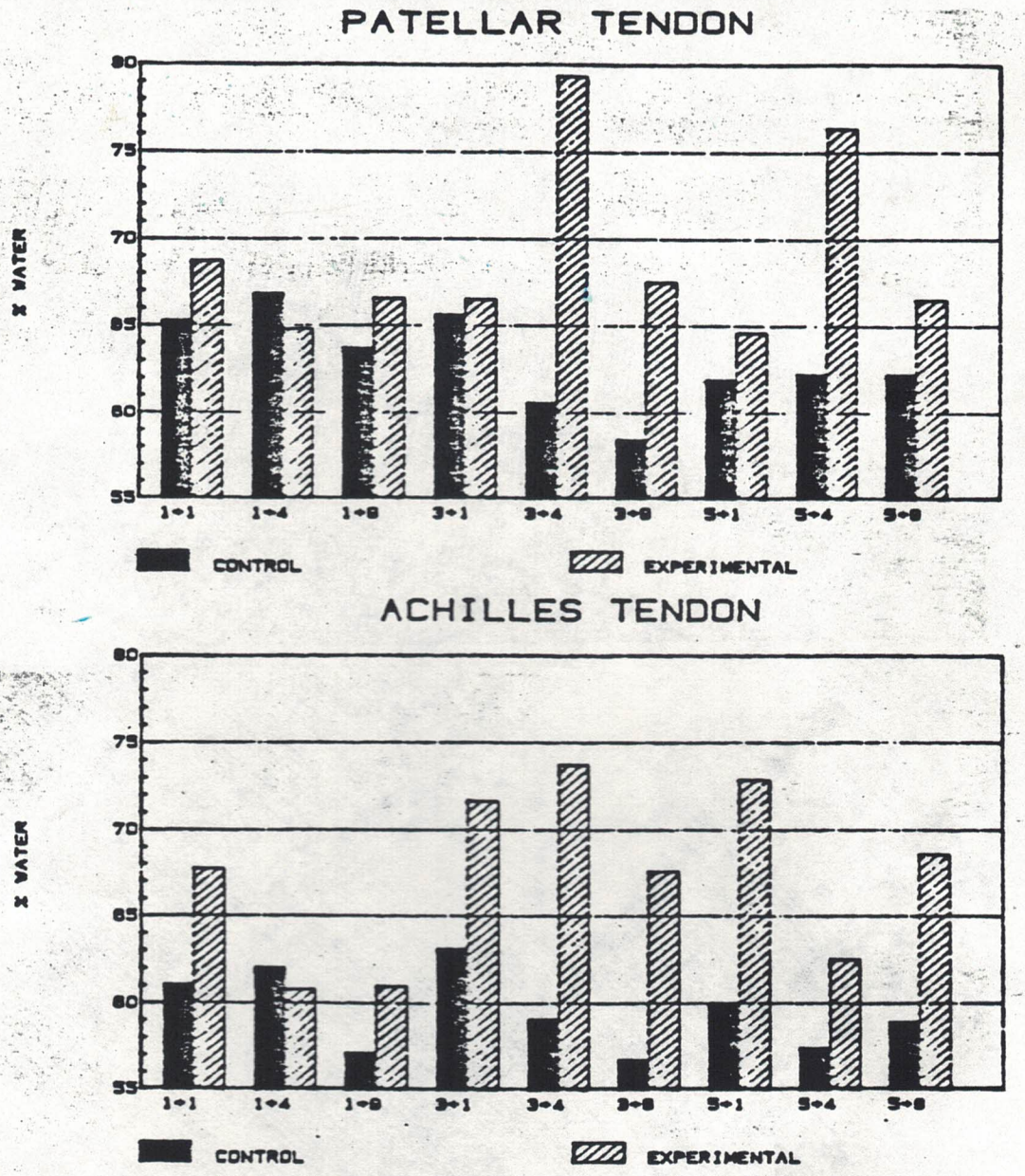

FIG. 7. Water content in patellar tendons and Achilles tendons.

AT. Mean values for the experimental tissues were not determined due to the variations in the number of injections and time periods.

DISCUSSION

The primary purpose of the present investigation was to see if we could gain insight into some of the underlying mechanisms that might be related to the increased separation force that Liu et al. (13) previously found in sodium morrhuate injected MCL.

As a sclerosing agent, the drug produces fibrous tissue buildup and is widely used for the obliteration of varicose veins where it produces its effect by irritation of the intimal endothelium. In addition to the treatment of varicose veins, Sugawa et al. (22) used sodium morrhuate to treat experimentally induced esophogeal varices in dogs and found the drug to be an effective thrombosing agent that produced perivenous fibrosis and thickening of the intima. The drug has also been used successfully in the management of hemangiomas (15), and in 1977

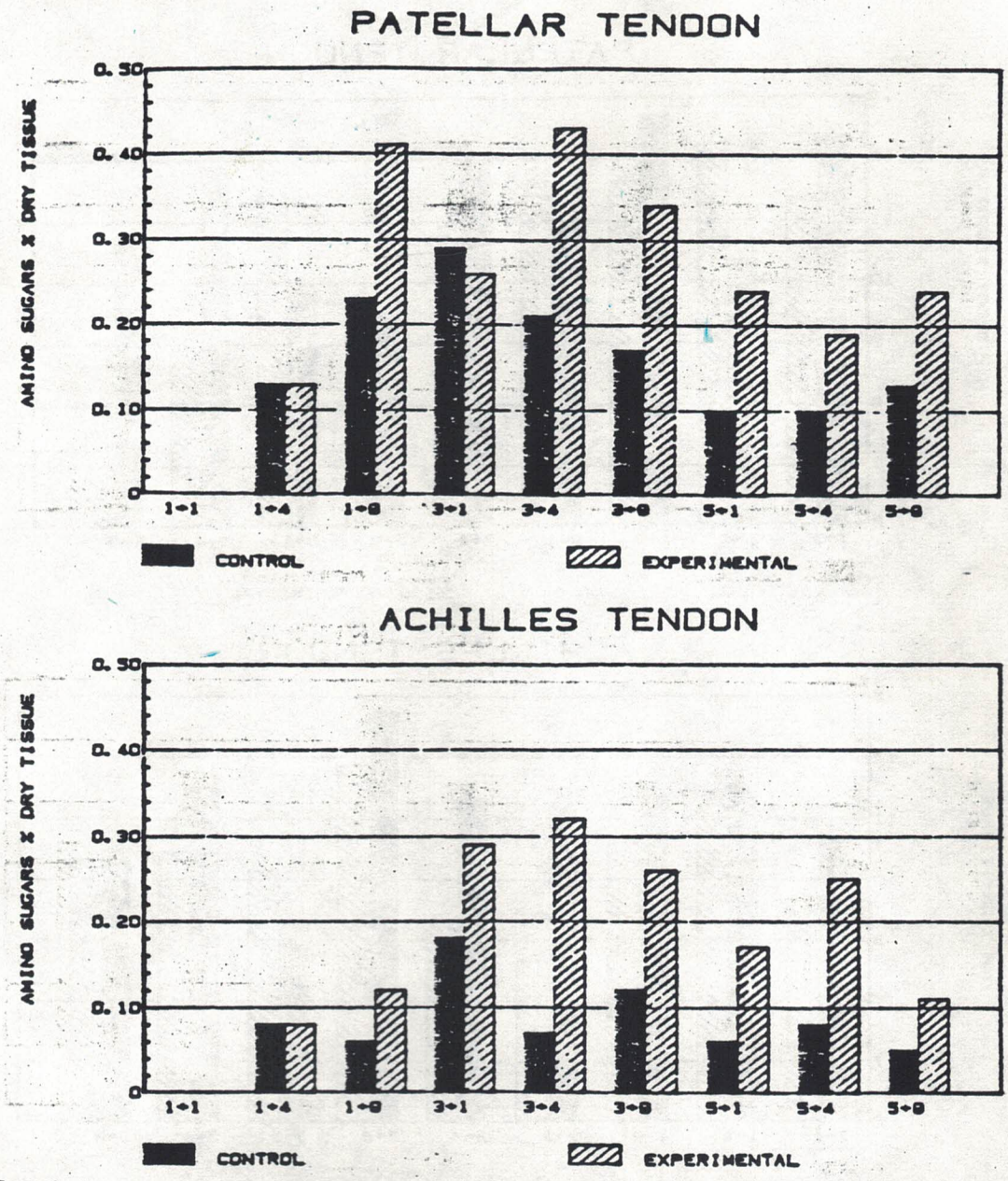

FIG. 8. Amino sugar (percent glycosamine plus galactosamine) content in patellar tendons and Achilles tendons.

Kastner and Wessel (11) treated 170 rheumatoid arthritis patients with sodium morrhuate (Varicocoid). Of the 231 joints treated, 107 were free from symptoms 12 months after treatment and an additional 76 were markedly improved. Morphological studies of the synovial membranes obtained after sodium morrhuate therapy indicated an early necrosis of lining cells and adjacent subsynovium, followed by an inflammatory reaction and later organizing fibrosis. Because of the extensive fibrosis that has been a consistent feature following sodium morrhuate injections, Liu et al. investigated the effects of the drug on ligamentous tissue strength and we conducted the current investigation to see if we could gain an understanding of how the drug exerts its influence.

Sodium morrhuate alters the morphometric features of tendons as demonstrated by an increase in the gross circumference of the structure and by differences in the collagen fibril sizes of injected versus control tissues. The increase in circumference appears to be due to an increase in cell pop-

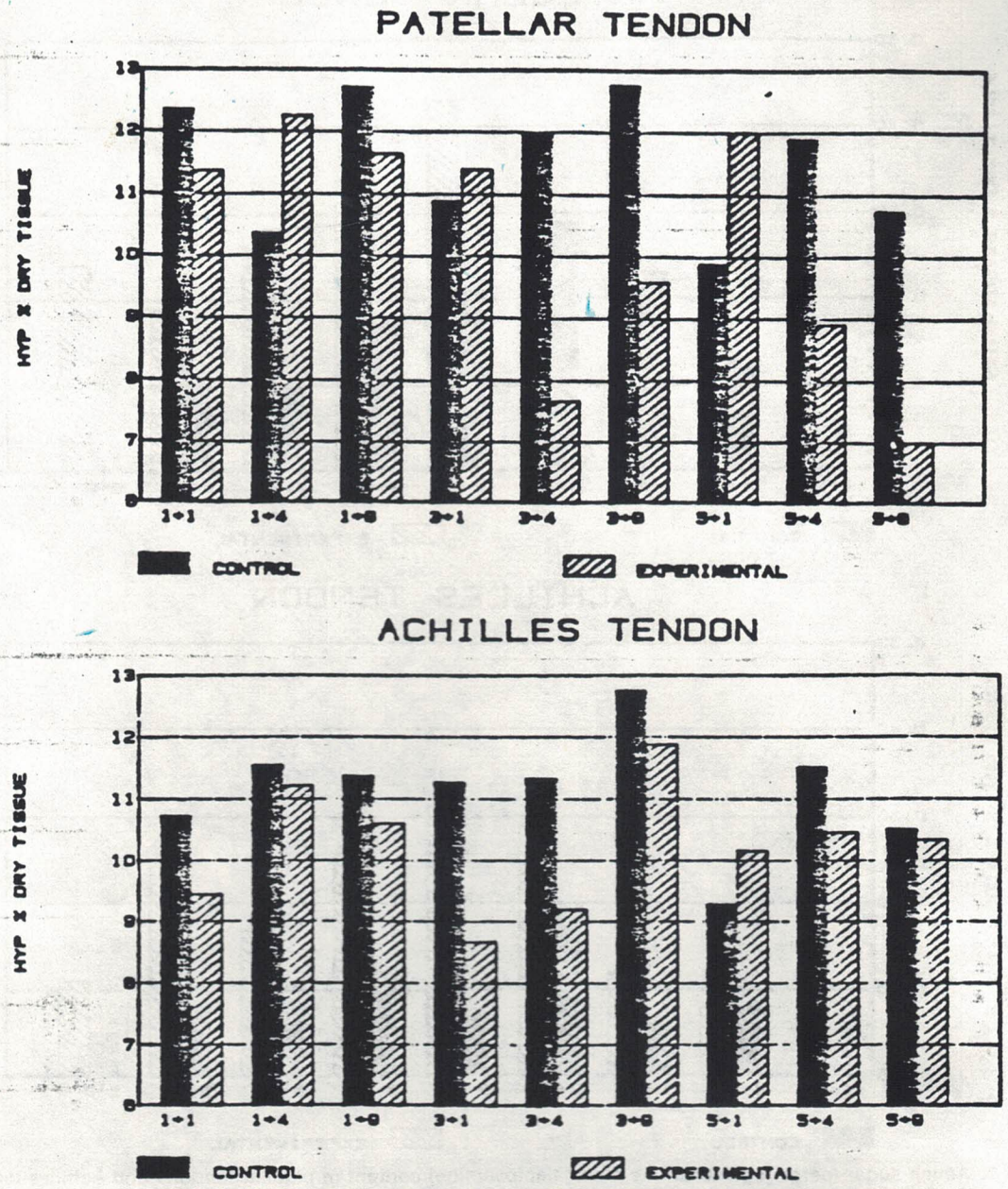

FIG. 9. Hydroxyproline content in patellar tendons and Achilles tendons.

ulation, water content, and ground substance. The increased cell population is a common characteristic of other studies using sodium morrhuate and apparently is due to the formation of granulation tissue in the injected area. Consequently, not only is there an increase in the number of cells but also a wider variety of cell types, fibroblasts, neutrophils, lymphocytes, plasma cells, and unidentifiable cells in the injected tissues. Interestingly, these findings are similar to dense connective tissues undergoing an injury-repair cycle in which the proliferation of cells from surrounding areolar tissue followed by formation of granulation tissue has been well documented (3,5,7,9,10,18,19). This is followed by fibroblast proliferation, increased vascular supply, and the deposition of new collagen. The trauma produced by the penetrations of the needle alone could not have triggered an injury-repair cycle in the present study since the opposite limbs in each animal received an equal number of control injections. The response was therefore due to the sodium morrhuate.

The increase in water content in the injected tendons paralleled the increase in cell content and therefore appeared to be due primarily to the increased cell population.

The amino sugar content was increased in nearly all the experimental tissues and indicates an increase in ground substance which may contribute to some extent to the enlarged experimental tissues and increased water content. The dominant hexosamine was galactosamine, suggesting an increase in chondroitin sulfate or dermatan sulfate in the tissues. Specific glycosaminoglycan (GAG) species were not identified; however, some glucosamine was also present in all the tissues and was consistently higher in the sodium morrhuate injected specimens (data not shown).

In injury-repaired ligaments Frank et al. (9) recently reported isolated areas of cartilage formation in some ligaments at 42 days following repair. Presumably, such sites could contain some keratan sulfate, which could contribute small levels of glucosamine such as were found in our specimens. Since we did not detect the presence of any chondrocytes in the histological sections of sodium morrhuate injected tendons, this tends to exclude the presence of any keratan sulfate in our specimens. Moreover, our amino sugar data were obtained after CPC treatment, which does not precipitate keratan sulfate. Therefore the increased glucosamine content observed in the sodium morrhuate injected tendons must have been due to the hyaluronate.

Further evidence of increased GAG content was indicated by the RR cytochemical reaction for proteoglycan-collagen relationships seen by electron microscopy. Both the size and number of granules were increased in some of the experimental specimens, particularly shortly after three and five injections. Luft (14) originally demonstrated the dense granules to indicate proteoglycans, and Laros and Cooper (12) later used the technique to demonstrate the location of proteoglycan aggregates along collagen fibrils. Our findings are identical to those of Laros and Cooper in terms of location and the specimens demonstrating an increase in size and number were those with a high amino sugar content.

The importance of proteoglycans in ligaments or tendon healing has not been investigated although Frank et al. (8) recently demonstrated an increased GAG content early in the healing process of MCL but did not illustrate this cytochemically. Hence the organization and location of the increased GAG content in repaired tissues have not been described. The present study indicates that proteoglycan content in collagenous tissue can be increased and is associated with collagen fibrils as previously described. Conceivably increased ground substance content could relate to increased ligament-bone separation force as found previously in sodium morrhuate injected MCL (13); however, such correlations remain to be demonstrated.

Amiel et al. (1) reported values for hexosamine content in PT and AT that are somewhat higher than ours. The difference may be due to the fact that our data were obtained after papain digestion and partial purification (CPC precipitation) of the GAGs, rather than by direct hydrolysis of the whole tissue. Their results agree with ours in indicating a higher GAG content in PT compared with AT.

The collagen fibril size frequency distributions were markedly different in the sodium morrhuate injected specimens, reflecting a high percentage of very small fibrils. These findings are interesting in relation to previous investigations concerning factors that determine collagen fibril size. These factors are age (16,17), mechanical requirements of the tissue (16,23), degree of glycosylation (2,20), and ground substance quantity and composition (4,6, 16,21). In the present study we found an increase in amino sugars that agrees with previous findings indicating GAG concentration retards fibrillogenesis, and collagenous tissues with high carbohydrate concentrations have small collagen fibril diameters (2,4,20,21). In developing rat tail tendon Scott et al. (21) found newly formed tendons to consist of small-size collagen fibrils and to be rich in chondroitin sulfate and hyaluronate. As development progressed, collagen fibril sizes increased dramatically as chondroitin sulfate decreased and dermatan sulfate increased. Although we did not identify specific GAG species, the sodium morrhuate injected tendons did have increased amino sugar concentration, whch could be due to chondroitin sulfate and hyaluronate. Therefore, the injected tendons appear to resemble newly formed tissue such as occurs in an injury-repair response. If this is correct, it may explain the abundance of small collagen fibrils in the sodium morrhuate injected tendons compared with controls. Aging cannot be considered a factor in our study, since the length of the study was not sufficient to be a factor and the collagen fibril size of control specimens was similar at all ages. Similarly, mechanical requirements would

be assumed identical for each limb since the rabbits did not show any impairment of movement of the sodium morrhuate injected limbs.

Finally, collagen content was proportionally lower in nearly every sodium morrhuate injected specimen. Frank et al. (9) found total collagen to be 79% in intact MCL but this value dropped markedly to a low value near 68% 21 days after injury. From this low value, the collagen content slowly progressed toward normal values but had not reached normal values by 100 days following injury. Converting hydroxyproline to total collagen, both of our experimental tissues averaged 72.8% compared with 79.5 and 82% for the control AT and PT, respectively. This mean value of 72.8% in our experimental tissue is close to the value reported by Frank et al. during ligament repair from 20 to 100 days postinjury and is inversely related to water, hexosamine, and cell content of the tissues.

From these results it appears that sodium morrhuate mimics an injury-repair response in connective tissue. There is an early formation of granulation tissue, cellular hyperplasia, increase in water and amino sugar content, and a decrease in collagen fibril diameter and hydroxyproline content compared with control tendons. The decreased mean collagen fibril diameter appears to be due to the formation of new collagen fibrils as evidenced by an increased cell population and a more active appearing organelle network within the fibroblasts. The size of the newly formed fibrils may be limited by an increase in GAG components of the tissue. Whether or not sodium morrhuate may be an effective agent to hasten repair responses in connective tissue remains to be determined.

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REFERENCES

1. Amiel D, Frank C, Harwood F, Fronek J, Akeson W: Tendons and ligaments: a morphological and biochemical comparison. *J Orthop Res* 1:257–265, 1984
2. Anderson JC, Labedz RI, Kewley MA: The effect of bovine tendon glycoprotein on the formation of fibrils from collagen solutions. *Biochem J* 168:345–351, 1977
3. Birdsell DC, Tustanoff ER, Lindsay WK: Collagen production in regenerating tendon. *Plast Reconstr Surg* 37:504–511, 1966
4. Birk DE, Lande MA: Corneal and scleral collagen fiber formation in vitro. *Biochim Biophys Acta* 670:362–369, 1981
5. Bora FW Jr, Lane DM, Prockop PJ: Inhibitions of collagen biosynthesis as a means of controlling scar formation in tendon injury. *J Bone Joint Surg [Am]* 54:1501–1508, 1972
6. Borcherding MS, Blacik LJ, Sittig JW, Bizzeu MB, Weinstein HG: Proteoglycans and collagen fibre organization in human corneoscleral tissue. *Exp Eye Res* 21:59–70, 1975
7. Clayton ML, Weir GJ: Experimental investigations of ligamentous healing. *Am J Surg* 98:373–378, 1959
8. Frank C, Amiel D, Akeson WH: Healing of the medial collateral ligament of the knee: a morphological and biochemical assessment in rabbits. *Acta Orthop Scand* 54:917–923, 1983
9. Frank C, Schachar N, Dittrich D: Natural history of healing in the repaired medial collateral ligament. *J Orthop Res* 1:179–188, 1983
10. Inglis AE, Sculco TP: Surgical repair of ruptures of the tendo Achilles. *Clin Orthop* 156:160–169, 1981
11. Kastner P, Wessel G: Chemical synovectomy with Varicocoid in rheumatoid arthritis—further results. *Scand J Rheumatol* 6:28–32, 1977
12. Laros GS, Cooper RR: Electron microscopic visualization of proteinpolysaccharides. *Clin Orthop* 84:179–192, 1972
13. Liu YK, Tipton CM, Matthes RD, Bedford TG, Maynard JA, Walmer HC: An in situ study of the influence of a sclerosing solution in rabbit medial collateral ligaments and its junction strength. *Connect Tissue Res* 11:95–102, 1983
14. Luft JH: Electron microscopy of cell extraneous coats as revealed by ruthenium red staining. *J Cell Biol* 23:54A, 1966.
15. Morgan JF, Schow CE: Use of sodium morrhuate in the management of hemangiomas. *J Oral Surg* 32:363–366, 1974
16. Parry DAD, Barnes GRG, Craig AJ: A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. *Proc R Soc Lond [Biol]* 203:305–321, 1978
17. Parry DAD, Craig AS, Barnes GRG: Tendon and ligament from the horse: an ultrastructural study of collagen fibrils and elastic fibers as a function of age. *Proc R Soc Lond [Biol]* 203:293–303, 1978
18. Peach R, Williams G, Chapman JA: A light and electron optical study of regenerating tendon. *Am J Pathol* 38:495–513, 1961
19. Postacchini F, Accinni L, Natali PG, Ippolito E, DeMartino C: Regeneration of rabbit calcaneal tendon: a morphological and immunochemical study. *Cell Tissue Res* 195:81–97, 1978
20. Schofield JP, Freeman IL, Jackson DS: The isolation and amino acid and carbohydrate composition of polymeric collagens prepared from various human tissues. *Biochem J* 124:467–473, 1971
21. Scott JE, Orford CR, Hughes EW: Proteoglycan—collagen arrangements in developing rat tail tendon. An electron microscopical and biochemical investigation. *Biochem J* 195:573–581, 1981
22. Sugawa C, Okumura Y, Lucas CE, Walt AJ: Endoscopic sclerosis of experimental esophageal varices in dogs. *Gastrointest Endosc* 24:114–116, 1978
23. Svoboda ELA, Howley TP, Deporter DA: Collagen fibril diameter and its relation to collagen turnover in three soft connective tissues in the rat. *Connect Tissue Res* 12:43–48, 1983
24. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch Biochem Biophys* 93:440–447, 1961