

## Biomechanical Properties of Normal Tendons, Normal Palmar Aponeuroses and Palmar Aponeuroses from Patients with Dupuytren's Disease Subjected to Elastase and Chondroitinase Treatment

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Normal tendons, normal palmar aponeuroses and specimens from patients with Dupuytren's disease, namely apparently normal palmar aponeuroses and contracture bands were subjected to elastase and chondroitinase ABC digestion. Maximum Young's modulus, normalized hysteresis loop and residual elongation were determined before and after enzyme treatment. In normal tendons, normal and apparently normal palmar aponeuroses both normalized hysteresis loop and residual elongation increased significantly after elastase treatment, whereas the stiffness decreased. Normalized hysteresis loop and residual elongation display changes corresponding to the amount of digested elastin. The increased viscosity of untreated contracture bands containing less elastin, as compared to normal palmar aponeurosis, was not affected by elastase. Obviously, the elastic fibers in normal shape and distribution are responsible for maintaining an elastic status with a low viscous stress component. With the exception of contracture bands chondroitinase ABC caused a minor increase of residual elongation and as opposed to elastase a decrease of normalized hysteresis loop indicating an increased mobility of the tissue fibers.

**Key Words:** Dupuytren's disease, palmar aponeurosis, biomechanics, elastase, chondroitinase ABC

### INTRODUCTION

Dupuytren's disease (DD) affects the dense connective tissue of the palmar aspect of the hand and the plantar aspect of the foot. The disease is characterized by alterations of the connective tissue fibers, proliferation of fibroblasts originating from perivascular areas, and neosynthesis of collagen. These alterations lead to a formation of dense strands of connective tissue (contracture bands, CB). DD is, therefore, classified as a fibromatosis. Vernon Luck<sup>1</sup> attributed the onset of DD to an active fibroblast proliferation. In contrast, Millesi described changes of the collagen fiber bundles, which precede the cellular proliferation. These "early changes" comprise thickening and fusing of collagen fibers as well as changes in thickness and distribution of the elastic fibers.<sup>2,3</sup> In addition a change of the composition of the proteoglycans is characteristic for DD tissue (decrease of hyaluronic acid and increase of chondroitin sulfate and dermatan sulfate).<sup>4</sup>

Mechanical studies concerning the viscoelasticity (re-

sidual elongation, relaxation and hysteresis loop) of specimens from patients with DD revealed a characteristic increase of the viscous stress component.<sup>5</sup> In apparently normal palmar aponeurosis of patients with DD (ANPA) the residual elongation is increased as compared to specimens of normal palmar aponeurosis (NPA) obtained from patients with carpal tunnel syndrome. Thus, mechanical changes are apparently present before the fibroblast proliferation occurs. In CB both residual elongation and hysteresis loop are dramatically increased when compared with ANPA. In a previous paper<sup>6</sup> we studied the effect of elastase digestion on the biomechanical parameters of NPA. We observed a sharp increase of the viscous fraction of the stress component measured by hysteresis loop and residual elongation after enzymatic treatment.

The aim of the present investigation was to study the effect of elastase and chondroitinase ABC on the mechanical properties of the following tissues:

1. Normal tendon (NT)
2. Normal palmar aponeurosis (NPA)
3. Apparently normal palmar aponeurosis (ANPA) from patients with Dupuytren's disease (DD)
4. Contracture bands from patients with DD
5. Rat tail tendons (RTT)

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## MATERIALS AND METHODS

### Specimens

Specimens of NT (of the palmaris longus muscle) and NPA were obtained during surgery of carpal tunnel syndrome. ANPA and CB were obtained at surgery of patients with DD. The number of patients together with the classification of specimens are given in Table I. RTT were obtained from adult Sprague-Dawley rats. The tendons were dissected free and single tendon fibers were pulled from the proximal end of the tail avoiding any irreversible straining.

### Elastase Treatment of Tissue Samples

10 mg saline-washed tissues were suspended in 1.0 ml phosphate buffered saline (PBS, pH 7.2) containing 10 units of pancreatic elastase purified by affinity-chromatography to reduce nonspecific protease contamination (Sigma E 0258, St. Louis, MO, USA). According to the manufacturer the elastase activity of the enzyme was 86 U/mg protein, 1.7 U/mg trypsin. Digestion of elastin was performed at 37°C for 3 hours. Following incubation the samples were centrifuged. Solubilized elastin peptides were determined using the Lowry method<sup>7</sup> (Folin reagent). Soluble elastin from ligamentum nuchae was used as standard. In addition we determined the hydroxyproline content of solubilized peptides using the chloramine-T method described by Stegemann and Stalder.<sup>8</sup> The efficiency of elastin digestion was verified by electron microscopic analysis.<sup>6</sup> Moreover, we determined the activity of elastase using elastin-congo red as substrate (Sigma; E-0502). When comparing digestion of elastin by pancreatic elastase in the presence or absence of soybean trypsin inhibitor (SBTI, Sigma, Type I-S, 0.1 mg per ml) using tendon as substrate we could not detect any significant difference in the amount of the tissue solubilized. However, a significant tryptic activity was observed if 0.001 M benzoylarginine ethyl ester was used as substrate in the absence of SBTI. This activity was completely inhibited by adding SBTI (0.2 mg/ml).

TABLE I

Number of patients and classification of tissue samples

		NT	NPA	ANPA	CB
Elastase	P	22	20	15	16
	S	25	23	15	19
Chondroitinase ABC	P	22	13	15	6
	S	25	13	15	8

Abbreviations: NT, normal tendon; NPA, normal palmar aponeurosis; ANPA, apparently normal palmar aponeurosis; CB, contracture band; P, patients; S, samples.

### Chondroitinase ABC Treatment of Tissue Samples

Tissue specimens were washed in PBS and transferred to 1 ml PBS containing 1 U of chondroitinase ABC from *Proteus vulgaris* (C-2905; Sigma). The samples were kept at 37°C for 24 hours. Following incubation tissue aliquots were used for biomechanical testing and analysis of glycosaminoglycans (GAG). The GAG content of enzyme-treated samples was compared to that of control samples incubated in the absence of chondroitinase ABC under the same conditions.

Small tissue samples were cut into pieces (<1 mm<sup>3</sup>) and the material was transferred to plastic vials, containing 0.5 ml 4 M guanidinium hydrochloride. The vials were incubated at room temperature for 18 hours under continuous agitation. The resulting supernatants were dialyzed against PBS in order to remove the guanidinium hydrochloride. The dialyzed fractions were treated with immobilized papain for 30 min at 37°C (P-4406; Sigma) and the digested supernatants recovered after centrifugation of the agarose beads. The fractions were stored at -20°C.

Analysis of the GAG composition of the samples was carried out by applying the samples to a high pressure liquid chromatography system consisting of a tandem arrangement of two separation columns (Biogel TSK 60 and 125; Biorad, Richmond, CA, USA) and a UV detector set to 214 nm wavelength. An isocratic solvent system with 4 M urea was used in all experiments. Peaks were recorded and integrated with a Chromatopac CR2A-integrator (Shimadzu, Tokyo, Japan) and the relative amounts of the individual GAG's expressed as percentage of the whole material as detected after separation. The individual peaks were compared with the results from separation of GAG standard samples (hyaluronic acid, chondroitin sulfate isomers, heparan sulfates; Sigma).

### Mechanical Tests

Strain controlled tests were performed at a rate of 1% per minute; i.e. within a frequency range of 10<sup>-3</sup>-10<sup>2</sup> Hz, which is characterized by low variations of dissipated energy.<sup>9</sup> The tests were carried out in a bath containing PBS. The clamps were manufactured from stainless steel. As padding material we used abrasive paper. The testing device was described in our previous paper on the role of elastin in normal palmar aponeurosis.<sup>6</sup> Strain was defined as the ratio of alteration in specimen length by original length, i.e. maximum length of a specimen under zero load. Length and alteration in length were measured with a potentiometer (maximum extension 100 mm, resolution 10 μm). Loads were measured with a load cell (maximum load 20 N, resolution 1 mN). Strain and loads were plotted with a X-Y graph recorder and then digitized. Loads were converted into "stress" by calculating the ratio load by

collagen content per unit length of the specimen. This method of normalization of loads to functional cross sectional area was described by Oxlund *et al.*<sup>10</sup> For comparison of enzyme-treated and control specimens we used the "stress"-strain relationship, normalized hysteresis loop, a measure of dissipated energy related to strain energy and the residual elongation. Normalized hysteresis loop was calculated as the ratio area between the loading and unloading graph by strain energy; residual elongation was measured after complete removal of load. Normalized hysteresis loop and residual elongation were measured after an unloading period starting from a strain value of 10%.

#### Statistical Analysis

The Kruskal Wallis test was used to detect overall effects, followed by Mann Whitney-tests for comparison of enzyme-treated and control specimens.  $p < 0.05$  was considered as statistically significant.

## RESULTS

#### Elastase Treatment of Tissue Samples

Our control experiments yielded the following results: 1: incubation of congo-red elastin with elastase resulted in the complete solubilization of the substrate (1 mg at 37°C for 3 h) and 2: elastase digestion of RTT did not result in the solubilization of collagen or other proteins, as verified by the determination of the hydroxyproline concentration and protein content of the supernatants.

In contrast, the enzyme solubilized significant amounts of protein from human tendons and palmar aponeuroses. The hydroxyproline content of the solubilized peptides was  $1.6 \pm 0.4\%$  (w/w), characteristic for elastin. When comparing the degree of elastin solubilized obtained by elastase treatment of different tissues we found differences in this solubility: the highest extractability was observed in NPA, the lowest in NT and ANPA as well as CB of DD patients showed intermediate elastin solubilities (Table II). The results of the statistical evaluation of these extraction experiments are shown in Table III.

As for the mechanical properties, RTT, NT, NPA and ANPA reveal low values of residual elongation and normalized hysteresis loop after unloading from 10% elongation (Fig. 1A, B and Tables V and VI). In CB, these parameters are significantly increased. Elastase digestion resulted in a significant increase of biomechanical parameters in NT, NPA and ANPA (Fig. 1A, B and Tables V and VI, row 2). Figures 2 and 3 indicate that changes in biomechanical parameters represent an approximately linear function of the elastin digested.

TABLE II  
Amount of elastin solubilized by elastase

Tissue	Relative amount of elastin peptides ( $\mu\text{g}/\text{mg d.w.}$ )
Normal tendon	$26 \pm 4.6$
Normal palmar aponeurosis	$62 \pm 14.2$
Apparently normal palmar aponeurosis	$42.3 \pm 5.3$
Contracture bands	$35.9 \pm 11.4$

#### Chondroitinase ABC Treatment of Tissue Samples

The relative amount of GAG degraded by chondroitinase ABC is shown in Table IV. It is interesting to note that CB from patients with DD display significantly less chondroitinase ABC sensitivity than NPA or ANPA. Compared to the effect of elastase the alterations of mechanical properties induced by chondroitinase ABC were not as pronounced as the effect of elastase digestion. If subjected to chondroitinase ABC digestion the residual elongation was significantly increased for NT, NPA and ANPA whereas the hysteresis loops were significantly reduced. In CB, however, treatment with the enzyme did not result in significant alterations of biomechanical parameters (Fig. 4A, B and Tables V and VI, row 3).

#### Stress-Strain Relationship

Stress as a function of strain displays a nonlinear behavior during loading and unloading procedure. Within a range up to approximately 3–4% strain the stiffness increases for all tissues under investigation. At higher strains stress increases proportional to strain, i.e. the stiffness remains approximately constant. The unloading curves do not coincide with the loading phase indicating a dissipation of strain energy. The maximum Young's modulus, which is obtained from the linear portion of the stress-strain graph, shows the highest value for NT, intermediate values for NPA and CB, and lowest values for ANPA (Table VII).

Typical examples of stress-strain relationships for the different tissues which illustrate the biomechanical parameters mentioned above are depicted in Figures 5 and 6.

TABLE III  
Statistical evaluation of elastin digestion

	NT	NPA	ANPA
NPA	<0.01	—	<0.01
ANPA	<0.01	<0.01	—
CB	<0.05	<0.01	n.s.

Abbreviations: NT, normal tendon; NPA, normal palmar aponeurosis; ANPA, apparently normal palmar aponeurosis; CB, contracture band.

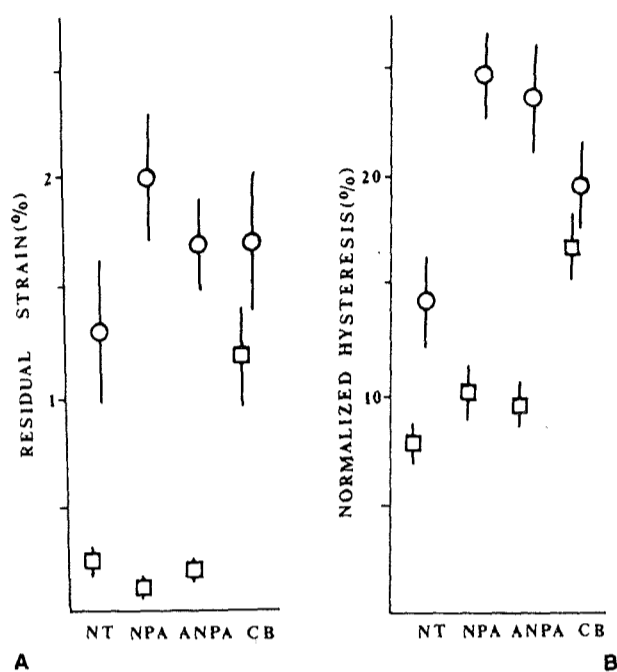


FIGURE 1 (A) Residual elongation (in % of original length) and (B) hysteresis loop (in % of strain energy) of controls (□) and elastase digested tissues (○). Abbreviations: NT normal human tendon, NPA normal palmar aponeurosis, ANPA apparently normal palmar aponeurosis, and CB contracture bands.

The highest Young's moduli were found for rat tail and human tendons and the lowest for ANPA, visible as differences in the steepness of the stress-strain relationships. This parameter is lowered significantly in response to elastase treatment except for CB.

## DISCUSSION

The normalized load-strain diagram for tendon is characterized by a nonlinear range up to strains of about 3% followed by a linear portion at higher strain values. It is well known from other studies<sup>11,12</sup> that the nonlinear segment of the curve corresponds to the straightening of the originally wavy arrangement of the collagen fibers. Minns *et al.*<sup>12</sup> found a remaining elongation of nearly zero after unloading tendons from strain levels up to 2.0%. We found a strain value of  $0.26 \pm 0.01\%$  remaining after complete unloading from a strain level of 10%, indicating the presence of a nonelastic component. As to the stiffness, NPA show a significantly lower maximum Young's modulus than tendon fibers indicating a higher extensibility. This is an astonishing finding considering the fact that the majority of the longitudinal fibers are extensions of the palmaris longus tendon. At a strain level of 10% used in the present paper the residual elongation of NPA did not differ from that of the macroscopically indis-

tinguishable ANPA (Table V). However, at strain levels of 2.5% and 5% used in a previous study<sup>5</sup> we detected significant differences. The residual elongation of CB was markedly higher than that of all the other tissue specimens. Since elastin has been shown to be responsible for the recoiling effect<sup>11,12</sup> this finding may be the consequence of a pathologically altered elastin network. The increase in the normalized hysteresis loop of CB as compared to the other tissues examined in our study (Table VI) can be explained by an enhanced friction suggesting an altered interaction of collagen fibers with the proteoglycan components.

To specify the biomechanical role of elastic fibers in tendons and palmar aponeuroses we used elastase digestion. The efficiency and specificity of the enzyme was checked using congo red elastin and rat tail tendons as substrates. Rat tail tendon, which is known to contain virtually no elastin,<sup>13</sup> showed neither biomechanical changes nor removal of collagen or any other proteins after elastase digestion. Therefore it can be concluded that no biomechanically relevant connective tissue components ex-

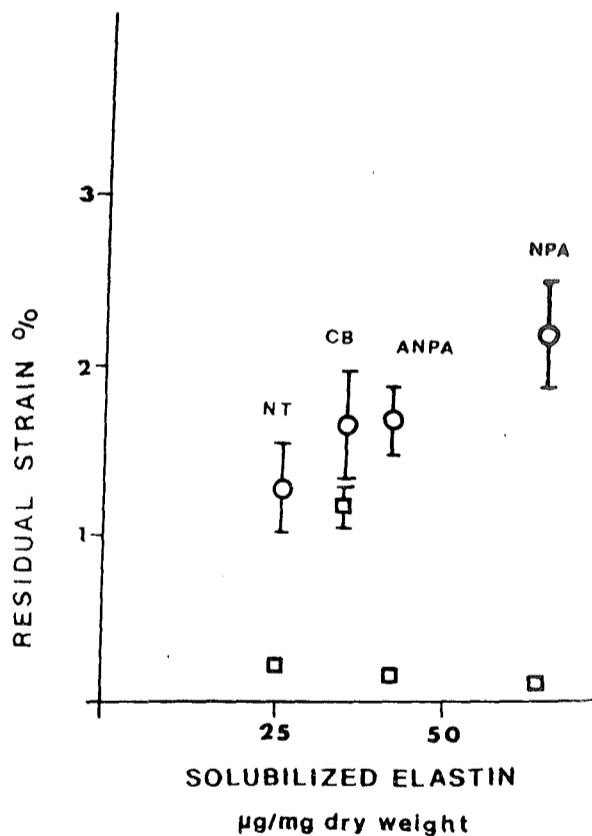


FIGURE 2 Linear relationship between residual strain (in % of original length) after elastase treatment (○) and the amount of solubilized peptides. Abbreviations: NT normal human tendon, NPA normal palmar aponeurosis, ANPA apparently normal palmar aponeurosis, and CB contracture bands. Controls: □.

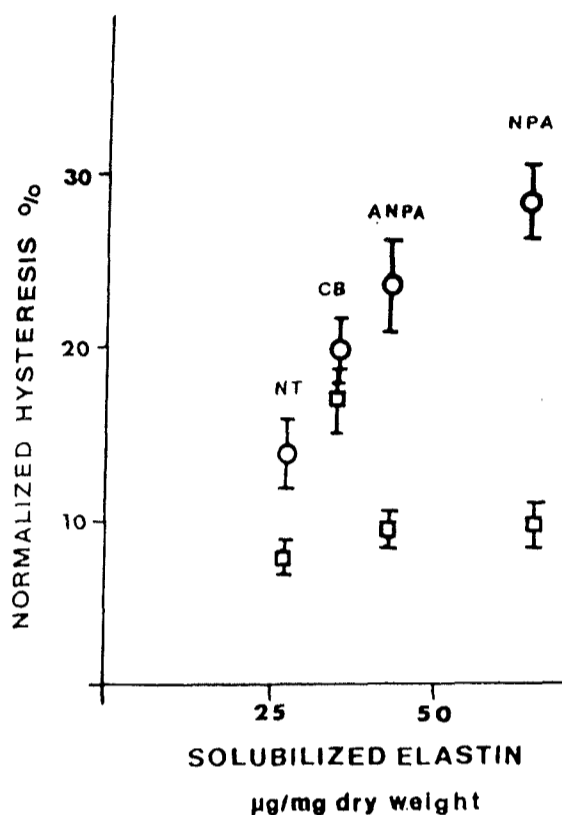


FIGURE 3 Linear relationship between hysteresis loop (in % of strain energy) after elastase treatment (○) and the amount of solubilized peptides. Abbreviations: NT normal human tendon, NPA normal palmar aponeurosis, ANPA apparently normal palmar aponeurosis, and CB contracture bands. Controls: □.

cept elastin, if present, are attacked by the enzyme. Using *human* tendons and palmar aponeuroses as substrates we observed low amounts of hydroxyproline concentration in the supernatants characteristic of solubilized elastin peptides.

The amount of solubilized elastin (Table II) corresponds to the relative abundance of elastic fibers, as detected in light microscopic studies.<sup>2</sup> Complete solubilization of elastic fibers after enzyme treatment is indicated by electron microscopic studies.<sup>6</sup> The residual strain of NT, NPA and ANPA increased significantly after elastase treatment. The increase was not significant in CB which

TABLE IV

Amount of glycosaminoglycans degraded by chondroitinase ABC	
Tissue	Relative amount (%)
Normal tendon	n.d.
Normal palmar aponeurosis	80 ± 9.9
Apparently normal palmar aponeurosis	81 ± 0.2
Contracture bands	59.7 ± 6.4

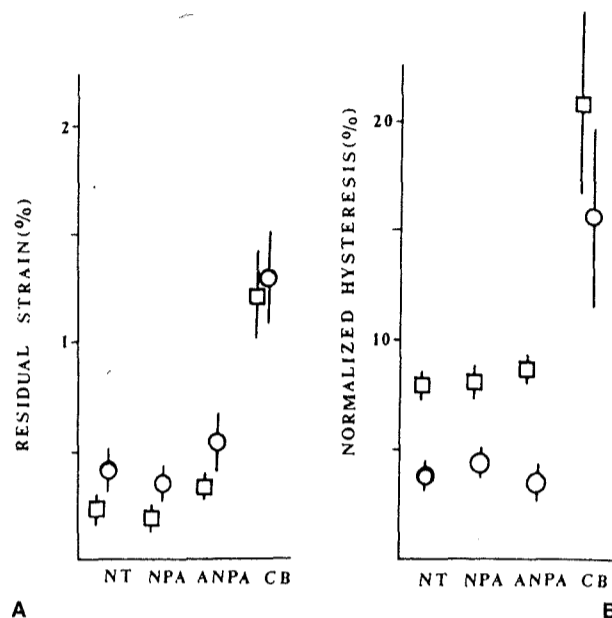


FIGURE 4 (A) Residual elongation (in % of original length) and (B) hysteresis loop (in % of strain energy) of controls (□) and chondroitinase ABC digested tissues (○). Abbreviations: NT normal human tendon, NPA normal palmar aponeurosis, ANPA apparently normal palmar aponeurosis, and CB contracture bands.

had a high residual elongation even before elastase treatment. As already mentioned the elastic fibers are responsible for the recoiling effect of tendons<sup>12</sup> and dermis.<sup>11</sup> It is obvious, therefore, that tissues with a functionally intact elastin network such as NT, NPA and ANPA show a low residual elongation. In these tissues residual elongation increases if the elastic fibers are eliminated by elastase. In CB, however, in which the elastic network is pathologically altered, elastase had no significant mechanical effect although a considerable amount of elastin was solubilized. Similar conclusions can be drawn from the influence of elastase treatment on the normalized hysteresis

TABLE V

Residual elongation of normal tendons, palmar aponeuroses and tissues from patients with Dupuytren's disease before and after enzyme treatment

Tissue	Residual elongation (%)		
	Controls	After enzyme digestion with	
		Elastase	Chondroitinase ABC
NT	0.23 ± 0.05	1.30 ± 0.32 <sup>§</sup>	0.40 ± 0.05 <sup>†</sup>
NPA	0.16 ± 0.03	2.20 ± 0.30 <sup>§</sup>	0.35 ± 0.07 <sup>†</sup>
ANPA	0.22 ± 0.04	1.70 ± 0.20 <sup>§</sup>	0.53 ± 0.12 <sup>§</sup>
CB	1.20 ± 0.20 <sup>†</sup>	1.70 ± 0.30	1.29 ± 0.20

Abbreviations: NT, normal tendon; NPA, normal palmar aponeurosis; ANPA, apparently normal palmar aponeurosis; CB, contracture bands. <sup>†</sup>*p* < 0.01 vs normal tendons; <sup>§</sup>*p* < 0.05; <sup>§</sup>*p* < 0.01 vs control tissue.

TABLE VI

Hysteresis loop of normal tendons, palmar aponeuroses and tissues from patients with Dupuytren's disease before and after enzyme treatment

Tissue	Hysteresis loop (%)		
	Controls	After enzyme digestion with	
		Elastase	Chondroitinase ABC
NT	7.90 ± 0.5	14.2 ± 2.0 <sup>‡</sup>	2.80 ± 0.3 <sup>‡</sup>
NPA	8.60 ± 0.8	28.5 ± 3.0 <sup>‡</sup>	4.40 ± 0.5 <sup>‡</sup>
ANPA	8.70 ± 0.9	23.9 ± 2.4 <sup>‡</sup>	3.70 ± 0.8 <sup>‡</sup>
CB	19.1 ± 2.8 <sup>†</sup>	19.9 ± 1.8	15.9 ± 4.2

Abbreviations: NT, normal tendon; NPA, normal palmar aponeurosis; ANPA, apparently normal palmar aponeurosis; CB, contracture bands. <sup>†</sup>*p* < 0.05 vs normal tendons; <sup>‡</sup>*p* < 0.01 vs control tissue.

TABLE VII

Young's modulus of normal, elastase and chondroitinase ABC treated tissues

	Controls	After enzyme digestion with	
		Elastase	Chondroitinase ABC
NT	2900 ± 280	2300 ± 200 <sup>†</sup>	3000 ± 250
NPA	1600 ± 300 <sup>‡§</sup>	980 ± 120 <sup>†</sup>	1700 ± 190
ANPA	1100 ± 250 <sup>‡</sup>	690 ± 75 <sup>†</sup>	1120 ± 100
CB	1600 ± 190 <sup>‡§</sup>	1250 ± 230	1550 ± 260

Abbreviations: NT, normal tendon; NPA, normal palmar aponeurosis; ANPA, apparently normal palmar aponeurosis; CB, contracture bands. <sup>†</sup>*p* < 0.05 vs control samples; <sup>‡</sup>*p* < 0.05 vs normal tendons; <sup>§</sup>*p* < 0.05 vs apparently normal palmar aponeuroses.

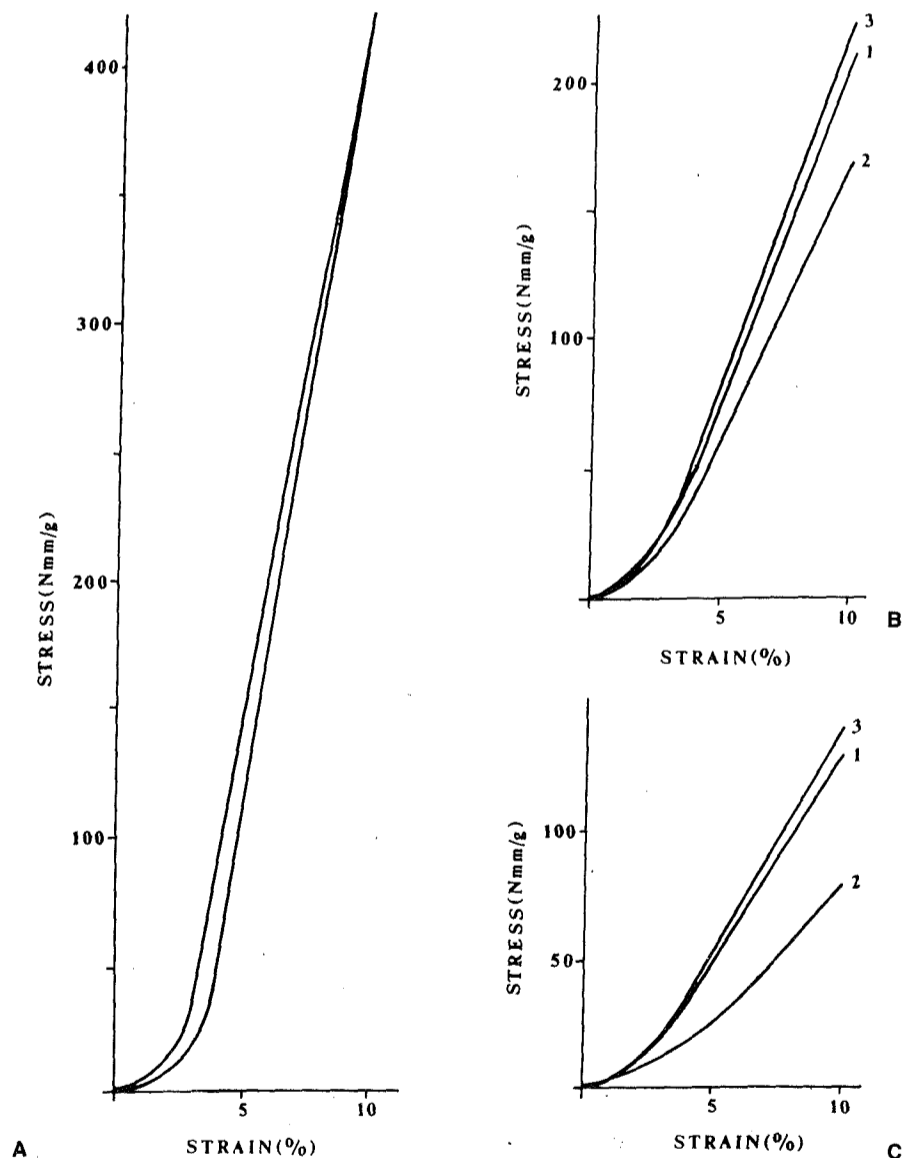


FIGURE 5 Stress (load normalized to collagen content per unit length) as a function of strain for (A) rat tail tendon, (B) normal human tendon, and (C) normal palmar aponeurosis. Curves (1) describe the stress-strain relationships of controls, curves (2) after elastase digestion and (3) after treatment with chondroitinase ABC.

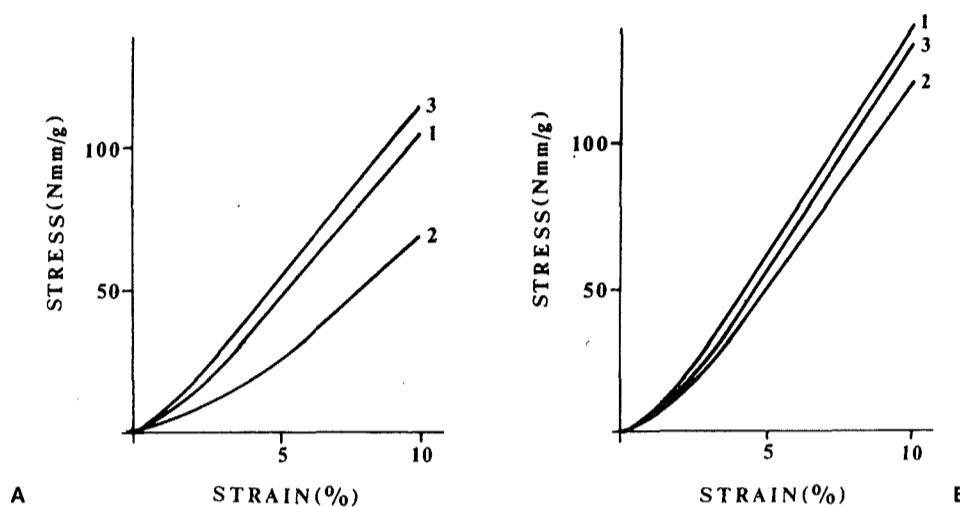


FIGURE 6 Stress (load normalized to collagen content per unit length) as a function of strain for (A) apparently normal palmar aponeurosis, and (B) contracture bands. Curves (1) describe the stress-strains relationships of controls, curves (2) after elastase digestion and (3) after treatment with chondroitinase ABC.

esis loop (Table VI). It seems evident that the elastic fibers are responsible for the low dissipated energy of NT, NPA and ANPA.

In order to study the biomechanical role of proteoglycans we used chondroitinase ABC to remove glycosaminoglycan side chains. More glycosaminoglycans are degraded in specimens from NT and ANPA than in CB, most likely due to better accessibility to the enzyme. In response to treatment with chondroitinase ABC the residual strain was slightly increased in ANPA and NPA (Table V). CB did not show any significant change in biomechanical parameters after chondroitinase ABC treatment. Therefore, glycosaminoglycans seem to play a minor role in the recoiling process. Normalized hysteresis loops display a uniform decrease in NT, NPA and ANPA, whereas again no alteration was detectable in specimens of CB. It can be concluded from these data that removal of at least a part of the glycosaminoglycans leads to a reduced interaction between the collagen fibers and the proteoglycans, resulting in a decrease of dissipated energy during load cycling.

In advanced cases of DD (CB) the amount of hyaluronic acid decreases and that of glycosaminoglycans chondroitin sulfate A and C increases when compared to NPA.<sup>14</sup> It would be of interest to know how chondroitinase ABC treatment changes the composition of proteoglycans under our experimental condition—a study is presently under way. The absence of a biomechanical effect of chondroitinase ABC treatment in CB seems to be related to the biomechanical predominance of the accumulated collagen fibers, concealing the minor mechanical effects of proteoglycans. The lack of a mechanical effect of elastase treatment on CB indicates that the elastic fibers present in CB do not contribute to the biomechanical behavior.

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