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THE ROLE OF NON-COLLAGEN COMPONENTS IN THE MECHANICAL BEHAVIOUR OF TENDON FIBRES

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SUMMARY

Determination of load–extension curves of rat-tail tendon fibres showed that when extension was restricted to 2% the slope of the linear portion of the curves was unchanged in successive tests. The change of slope resulting from enzymic treatment was used to assess the damaging effect of hyaluronidase and trypsin. Pure hyaluronidase had no significant effect indicating that chondroitin sulphate A and C and hyaluronic acid are not important in stabilising the fibres according to this criterion. Other preparations of hyaluronidase did damage the fibres and this is attributed to the presence of enzymic impurities which attack the interfibrillar matrix rather than the collagen of the fibres. Evidence is presented that it is the non-collagenous protein of the matrix that is attacked and which is important in stabilising the native fibres.

INTRODUCTION

For many purposes it is convenient to regard connective tissues as consisting of two more or less distinct extracellular fractions, the protein fibres and the amorphous matrix or ground substance in which they are embedded[1,2]. The way in which any particular connective tissue responds to applied stress will depend on the relative proportions of these two fractions and the way in which they interact. In tissues such as articular cartilage, where the matrix predominates, its importance in determining the mechanical behaviour of the material has long been recognised[2–6]. The matrix comprises only a small fraction of tendons, however and although the tensile properties of tendons and other collagenous fibres have been intensively studied[2,7–15], attempts to relate these properties to tissue structure have largely been concerned with the structure of the fibrous protein, collagen, itself and the role of the matrix has been somewhat neglected[16,17].

Acid mucopolysaccharides, which are the major organic constituents of connective tissue matrix, comprise about 0.5% of the weight of acetone-dried tendon[18]. Chondroitin sulphates B and C and hyaluronic acid (as a minor component) have been isolated from tendons[18,19] and the presence of neutral polysaccharide has also been reported[20,21]. The non-collagenous proteins of connective tissue have been less well characterised although they have been shown to be intimately associated with

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collagen prepared from skin[22] and bone[23]. Our knowledge of the macromolecular structure of connective tissue mucopolysaccharides is largely the result of study of material extracted from tissues such as cartilage where they are present in large amount. In cartilage the long unbranched chains of chondroitin sulphate are strongly bound to non-collagenous protein[24–27] and similar mucopolysaccharide–protein complexes have been extracted from other connective tissues[28].

Attempts have been made to assess the importance of mucopolysaccharides and non-collagenous protein in the properties of loose connective tissue[29,30] and uterine cervix[31] of the rat by observing the effects of degrading them with enzymes which have been assumed to leave the collagen intact. In the same way mucopolysaccharides have been shown to play a part in the mechanical behaviour of elastic tissue[32,33]. In the present work we have investigated the use of a similar approach to the study of the part played by mucopolysaccharide and non-collagenous protein in the tensile properties of rat-tail tendon fibres.

EXPERIMENTAL

Materials

Tendon fibres: The tails of adult rats (Wistar) were dissected immediately after sacrifice and fibre bundles (approx. 0.3 mm diameter) extracted with the minimum of stretching. They were washed overnight in sterile isotonic saline solution at 0.4°, stored in the same medium and used within one week.

Chondroitin sulphate–protein complex: The complex was prepared from fresh bovine nasal septum by high speed homogenisation with water[26]. It contained 24.2 % hexosamine and 0.40 % hydroxyproline (corresponding to 3.3 % collagen).

Enzymes: The following commercial preparations of testicular hyaluronidase were used: bovine, L. Light & Co. Ltd.; bovine, British Drug Houses Ltd.; ovine, L. Light & Co. Ltd.; ovine, Bengers Ltd. They are referred to below as preparations A, B, C and D, respectively. Trypsin was a twice-recrystallised preparation (L. Light & Co. Ltd.).

Protein solutions: Solutions of acid-soluble calf-skin collagen were prepared by the method of WOOD AND KEECH[34] and dialysed against 0.23 M NaCl containing 0.02 M Tris buffer (pH 7.1) at 0–4°. Solutions (1 %) of gelatin were prepared in the same Tris-buffered 0.23 M NaCl at 37° and equilibrated at this temperature for 1 h before use. Two samples of gelatin were used; one was an acid pigskin gelatin (gelatin No. 149 (see ref. 35)) whose amino acid composition has been reported by EASTOE[36] and which, according to LEACH[35], does not contain any of the heat-coagulable mucoprotein present as a minor component (0.1 %) in some other gelatins; the other was a lime processed ox-hide gelatin (gelatin No. 127 (see ref. 35)) whose amino acid composition is also known[36] and from which a protein–polysaccharide complex (0.36 %) has been isolated[37]. Neither gelatin gave a distinct peak at 530 mμ in its absorption spectrum after treating with diphenylamine, a reaction which has been used by COURTS[38] to detect mucoproteins in gelatins.

METHODS

Load-extension curves of tendon fibres

The apparatus (Fig. 1) was designed to give load-extension curves at constant rate of strain. One end of the fibre (approx. 5 cm long) was held in a stainless steel

clamp suspended from a stiff steel spring whose ends were rigidly attached to a brass plate. The other end of the fibre was held in a similar clamp carried by a stainless steel bar which could be driven in a vertical direction by rotating a micrometer head, thus stretching the fibre. The displacement of the centre of the spring arising from the tension in the fibre was detected by a Philips displacement pick-up (P9310) mounted on the brass plate. The signal from the pick-up was fed to a Philips direct-reading measuring bridge (PR9300) which was balanced with the fibre unstretched. The meter gave readings which were proportional to load and was calibrated by hanging known weights from the spring in place of the fibre. The centre of the spring was displaced approx. 10 μ by a load of 120 g, *i.e.* about 0.02 % of the fibre length. Some difficulty was encountered in effectively gripping the ends of the fibres but small engineers' clamps with serrations 0.02 in deep on the faces were found to be suitable. Each jaw was lined with thin wash-leather in order to avoid damaging the fibres.

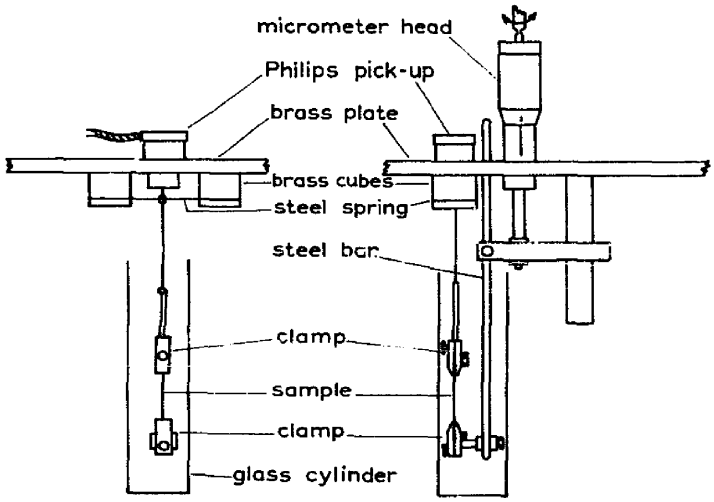

Fig. 1. Diagram of extensometer.

Fibres were chosen so that their diameters did not vary along their length by more than about 10 % of their mean diameter. In order to approach physiological conditions the fibres were immersed in 0.23 M NaCl containing either 0.02 M Tris buffer (pH 7.1) or 0.02 M sodium phosphate buffer (pH 7.1) in the glass cylinder (Fig. 1) which was itself immersed in a constant temperature bath. Before a load extension curve was determined the fibre was left in the apparatus for 15 min to attain swelling equilibrium and then drawn taut. The length of the fibre was measured with a travelling microscope reading to 0.002 cm. The fibre was extended by rotating the micrometer head either by means of a motor at a constant rate of 1 %/min, or manually, at about the same overall rate, in increments of 0.01 cm.

Enzymic treatment of fibres: Single fibres were incubated with 5 ml enzyme solution for 16 h at 35° or 37° and then washed with 3 changes of 5 ml buffered salt solution for 3 h. Control fibres were incubated with buffer alone. In order to avoid bacterial contamination all buffer solutions were autoclaved and penicillin (100 I.U./ml) and streptomycin (100 μg/ml) included in the incubation mixtures.

Fibre shrinkage temperature was determined by the method of JACKSON[39]. Enzymic degradation of collagen, gelatin and chondroitin sulphate–protein complex in solution was followed viscometrically. A known volume of chondroitin sulphate–protein complex (0.4 %) or gelatin (1 %) was equilibrated at 37°. A known volume of enzyme solution, also equilibrated at 37°, was added and after mixing, a known volume was transferred to a viscometer at 37° and the flow time determined at intervals during the reaction. Degradation of collagen at 25° was followed in a similar manner but $CaCl_2$ was incorporated into the enzyme solution so that its concentration in the reaction mixture was 0.5 M (see ref. 40). Ostwald-Fenske viscometers (10 ml) with flow times for water at 37° of about 4 min were used with gelatin. Miniature U-tube viscometers (2 ml) with flow times about 1 min for water at 37° were used with chondroitin sulphate–protein complex and similar viscometers, with flow times for water at 25° of about 2 min, were used with collagen.

RESULTS

Load-extension curves of native tendon fibres in buffer solutions (pH 7.1) at 25° were similar to those described by others for native fibres and fibres which have been dried and then re-swollen[8–12, 15]. They showed (Fig. 2) an initial region concave to the load axis followed by a linear region which persisted up to 3–5 % extension. Retraction curves showed considerable hysteresis but the original length was regained, provided the fibre had not been stretched more than 3 %.

When fibres were stretched more than 3 %, irreversible changes occurred and they did not return to their original length when released. Only occasional fibres could be stretched more than 4–7 % even when taken through a series of load extension cycles whose maximum extension increased in increments of 0.5 % (see ref. 15).

As observed by MORGAN AND MITTON[41] and HALL[11] there was considerable variation in the slope of the linear portion of stress–extension curves. This effect (Fig. 3) did not appear to be related to fibre diameter or length but was not investigated in detail.

Successive load extension curves up to 2 % extension for single fibres which were allowed to rest unstretched for 15 min between experiments were similar but showed a progressive displacement to higher extensions. The magnitude of this displacement varied from fibre to fibre and did not appear to be related to fibre diameter. When the experiments were done with the fibres immersed in phosphate-buffered NaCl solution, the slope of the linear portion of the curve also fell progressively but using Tris-buffered NaCl solution the slopes of successive curves were almost the same (Fig. 4) except in about 3 % of the fibres which showed progressive weakening. Tris-buffered NaCl solutions were used in all subsequent experiments.

Effect of enzymic treatment on load-extension curves

The rather wide variation of stress-extension curves of rat-tail tendon fibres from fibre to fibre would preclude any precise assessment of the effect of enzymic treatments based on comparison of the behaviour of treated and untreated fibres. However, since the slopes of the successive load-extension curves up to 2 % extension were reproducible it was considered likely that the effect of enzymes might be assayed by inter-

posing an enzymic treatment between the determination of two successive curves, when any change of slope could be attributed to the effect of the enzyme.

Hyaluronidase

Two load extension curves up to 2 % extension were determined on each of several tendon fibres. This enabled us to discard the few fibres (about 3 %) which behaved atypically. One group of fibres was then treated at 37° with hyaluronidase A (1 mg/ml

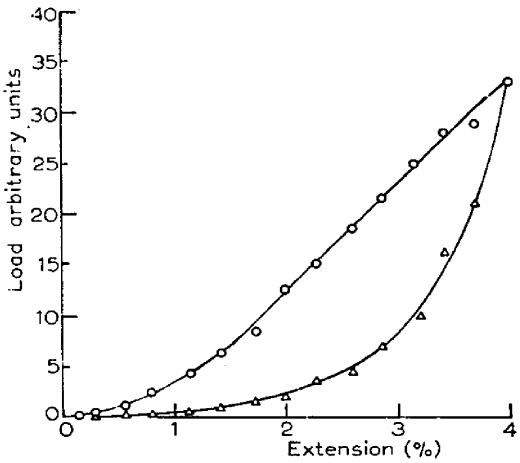

Fig. 2

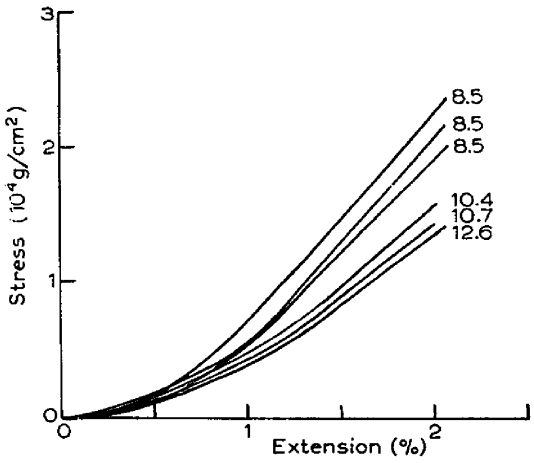

Fig. 3

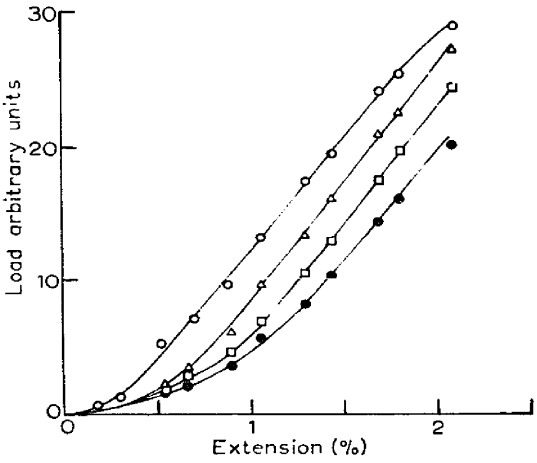

Fig. 4

Fig. 2. Typical load-extension cycle for a rat-tail tendon fibre immersed in 0.23 M NaCl containing 0.02 M Tris buffer (pH 7.1) at 25°. Rate of extension approx. 1 %/min: O—O extension, △—△, retraction.

Fig. 3. Stress-extension curves for several tendon fibres from a single rat tail. The figure accompanying each curve is the mean cross-sectional area (cm^2) × 10^4.

Fig. 4. Successive load-extension curves (in order, O—O, △—△, □—□, ●—●) for a tendon fibre immersed in Tris-buffered 0.23 M NaCl (pH 7.1) at 25°. Rate of extension approx. 1 %/min. Interval between extensions 15 min.

≡ 300 I.U./ml) and another incubated with buffer alone. Both groups were then retested. The results, of which those shown in Fig. 5 are typical, demonstrated that this enzymic treatment markedly reduced the slope of the load extension curves whilst the control group was almost unchanged. Most of the treated fibres did not recover their original length after the first post-incubation extension. Some of them broke before reaching 2 % extension. When treated fibres were stretched to break their fibrillar components appeared to slip over one another and did not break cleanly across, as untreated fibres did. The same behaviour was observed with fibres treated

with 0.1 mg/ml hyaluronidase A, variable results were obtained using an enzyme concentration of 0.01 mg/ml whilst no damaging effect was observed at 0.001 mg/ml.

TABLE I

EFFECT OF HYALURONIDASE (SAMPLE A) ON THE LOAD-EXTENSION CURVES OF TENDON FIBRES

S_1 and S_2 are the slopes of the linear portions of load-extension curves determined before and after incubation with enzyme or with buffer (controls).

pH	$(S_1 - S_2)/S_1$	
	Control	*Enzyme-treated*
4.9	0.16	0.14
5.5	0.00	0.93
6.2	0.27	0.89
7.1	0.00	0.92
7.5	0.10	0.73
8.0	0.15	0.00

Treatment with hyaluronidase A had the same damaging effect from pH 5.5 to pH 7.5 (Table I). The enzyme was inactive in a narrow region on each side of this pH range but the total pH range that could be covered was limited by the fact that the exposure of fibres to buffers of pH less than 4.4 or greater than 8.6 at 37° itself had a damaging effect.

Hyaluronidases B and C had a similar damaging effect to preparation A but were

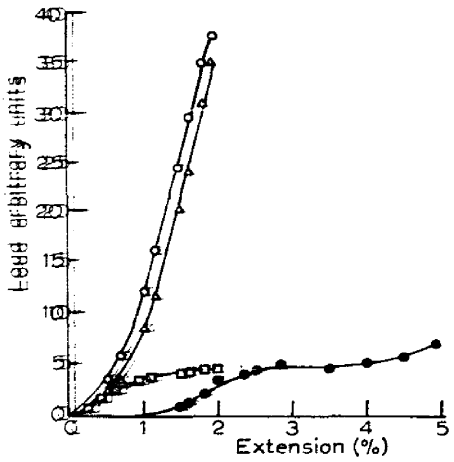

Fig. 5. Effect of treating a fibre with hyaluronidase (sample A) on its load extension curve. ○—○ and △—△, two successive curves before treatment. □—□ and ●—●, two successive curves after treatment. Details of enzymic treatment are given in text and comparison of treated and control fibres in Table I.

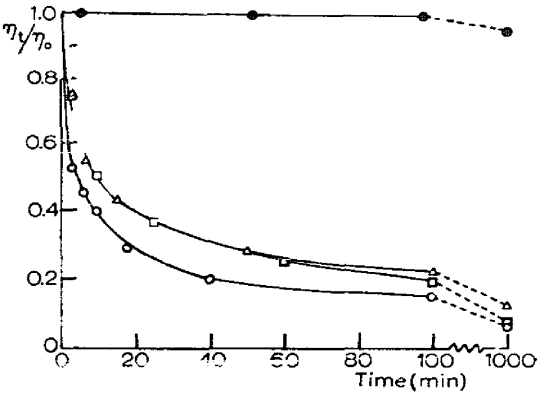

Fig. 6. Degradation of chondroitin sulphate-protein complex by hyaluronidase. η_0 and η_t are specific viscosities at 37° of mixtures of 3 ml 0.4% solution of chondroitin sulphate–protein complex and 0.5 ml hyaluronidase solution at time of mixing and at time t after mixing. ○—○, Hyaluronidase A; △—△, hyaluronidase D; □—□, hyaluronidase A, at final concentrations 50, 50, and 25 I.U./ml, respectively. ●—●, buffer (control).

not examined in detail. Hyaluronidase D, on the other hand, had a much smaller effect on the load-extension curve although it was studied over the pH range 5.8–8.1 (Table II).

TABLE II

EFFECT OF HYALURONIDASE (SAMPLE D) ON THE LOAD-EXTENSION CURVES OF TENDON FIBRES

S_1 and S_2 are the slopes of the linear portions of load-extension curves determined before and after incubation with enzyme of with buffer (controls).

pH	$(S_1 - S_2)/S_1$	
	Control	*Enzyme-treated*
5.8	0.03	−0.11
6.8	0.21	0.15
7.0	−0.21	−0.04
8.0	−0.05	0.00
8.1	−0.05	−0.11

Trypsin

This enzyme had a less marked damaging effect than any of the hyaluronidase preparations and was not studied in detail

Shrinkage temperature

Fibres treated with hyaluronidase A contracted in Tris-buffered saline in the same temperature range (59–61°) as fibres which had been incubated with buffer alone.

Degradation of chondroitin sulphate–protein complex by hyaluronidase and trypsin

All the preparations of hyaluronidase reduced the viscosity of a solution of

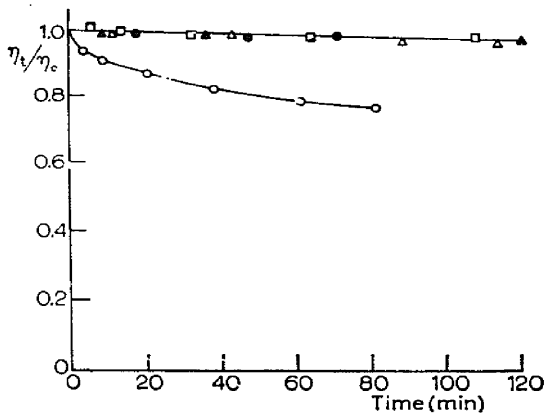

Fig. 7. Effect of hyaluronidase and trypsin on the viscosity of a collagen solution. η_0 and η_t are the specific viscosities at 25° of mixtures of 2 ml collagen solution, 0.3 ml 4 M $CaCl_2$ and 0.1 ml enzyme solution at time of mixing and at time t after mixing. O—O, trypsin; ▲—▲, △—△, □—□, hyaluronidase, samples A, C and D respectively; ●—●, buffer (control). Concentration of enzymes in reaction mixture 0.2 mg/m.

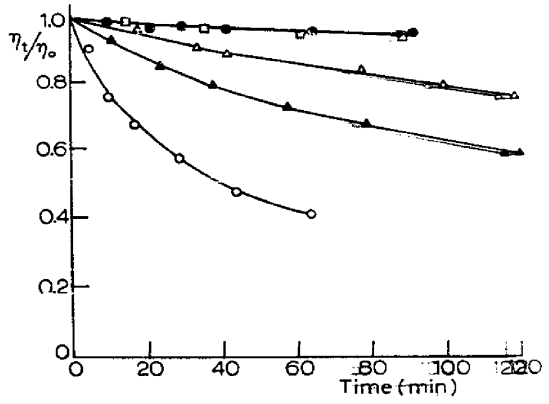

Fig. 8. Effect of hyaluronidase and trypsin on the viscosity of a gelatin solution (No. 149) η_0 and η_t are the specific viscosities at 37° of mixtures of 10 ml gelatin solution and 2 ml enzyme solution at time of mixing and at time t after mixing. Concentrations of enzymes in reaction mixture; O—O, trypsin (1.0 μg/ml); ▲—▲, trypsin (0.2 μg/ml); △—△, □—□, hyaluronidase, samples A and D respectively (50 μg/ml); ●—●, buffer (control).

chondroitin sulphate–protein complex (Fig. 6). However preparations A and C, which had a marked damaging effect on tendon fibres, did so more rapidly than preparation D which had only a very small damaging effect. Moreover, even when the concentrations of A and C were decreased so that their degradation of chondroitin sulphate–protein complex occurred at the same initial rate as that due to D, they reduced the viscosity to a greater extent than D indicating that degradation had proceeded to a greater extent.

Effect of hyaluronidase on protein solutions

Collagen: Hyaluronidase preparations A, C and D had no effect on the viscosity of a solution of collagen (pH 7.1) at 25° (Fig. 7). Trypsin, on the other hand, had a small though significant effect in agreement with HODGE *et al.*[42].

TABLE III

EFFECT OF HYALURONIDASE ON THE VISCOSITY OF GELATIN SOLUTIONS

$(\eta_{sp})_0$ and $(\eta_{sp})_{100}$ are the specific viscosities of the gelatin at time of addition of enzyme at 37° and 100 min later. Hyaluronidase concentration 50 I.U./ml and gelatin concentration 0.83 g/100 ml in reaction mixture.

Enzyme	$100[(\eta_{sp})_0 - (\eta_{sp})_{100}]/(\eta_{sp})_0$	
	Gelatin 149	*Gelatin 127*
None	9.2	4.0	3.6
Hyaluronidase A	23.8	21.4	14.0
Hyaluronidase B	—	9.1	11.8
Hyaluronidase C	—	14.9	18.2
Hyaluronidase D	11.4	4.4	2.8
Trypsin 0.2 μg/ml	39.4	—	—
Trypsin 1.0 μg/ml	64	—	—

Gelatin: Hyaluronidase A markedly reduced the viscosity of gelatin solutions (pH 7.1) at 37°, the magnitude of the effect being equivalent to the presence of about 0.1 μg/ml trypsin in the reaction mixture (Fig. 8). Hyaluronidase C behaved in a similar manner (Table III) but hyaluronidase D had an extremely small effect on the viscosity of gelatins. This effect of hyaluronidases A and C was not inhibited by $HgCl_2$ ($1.3 \cdot 10^{-3}$ M and 10^{-2} M), $CaCl_2$ (10^{-2} M), EDTA (10^{-2} M), soybean trypsin inhibitor (0.33 μg/ml) or by pre-treatment with *p*-chloromercuri benzoate.

DISCUSSION

The observed shape of load–extension curves of rat-tail tendon fibres is generally in agreement with the results of other workers with tendon fibres from this and other sources. Our observation that successive load-extension curves up to 2% extension are displaced along the extension axis is not, however, in accord with the observations of RIGBY *et al.*[15]; nor have we been able to confirm their finding of the conversion of the fibres into a highly extensible form merely by repeated stretching. However, in agreement with their work and with that of MORGAN AND MITTON[41] and HALL[11] the slopes of the linear portions of successive load extension curves of single fibres were found

to be nearly the same and this enabled us to show that treatment of the fibres with hyaluronidase damages them.

The repeated testing procedure provides, in principle, a method of assaying the effect of any treatment, which eliminates the effect of the very marked fibre-to-fibre variation of stress-strain curves. It was hoped that this would enable us to assay quantitatively the effect of enzymic treatment. The method could not be fully exploited, however, because of the fibre-to-fibre variation in the response of the material to low concentrations of the enzymes. In spite of this, the results clearly show the marked effect which certain preparations of testicular hyaluronidase have on the mechanical properties of the tendon fibres. Not only is the slope of the load extension curves much reduced but extension even to only 2% is to a large extent irreversible, *i.e.* pronounced plastic flow occurs, unlike untreated fibres which are perfectly elastic.

From the difference in manner of breaking of treated and untreated fibres it is concluded that the enzyme(s) attacks regions of the fibre lying between its components. The term "tendon fibres" which we have, for brevity, used to describe the experimental material is not entirely accurate, for each of our "fibres" is in reality a bundle of fibres each of which can in turn be dissected into still thinner fibres. This process could, presumably, be repeated down to the level of the collagen fibrils, those entities which are identifiable by their characteristic appearance in the electron microscope and are 500–2000 Å in diameter. The way in which treated fibres break indicates that the active hyaluronidase preparations attack inter-fibre and/or inter-fibril material usually referred to as matrix or ground substance.

The failure of hyaluronidase to affect the shrinkage temperature of the tendon fibres confirms and extends the observations of BROWN *et al.*[43] and indicates that the sites attacked by the enzyme do not lie in the collagen fibrils themselves. In addition, the failure of the enzyme preparations to reduce the viscosity of collagen solution under conditions which favour degradation by collagenase[40] and trypsin[42] also indicates that collagen itself is not attacked by hyaluronidase. Our results thus provide strong evidence for the importance of the integrity of the matrix for the mechanical strength of tendon fibres in the rat tail.

The actual site of action of these enzyme preparations is uncertain. Testicular hyaluronidase is considered to degrade hyaluronic acid and chondroitin sulphates A and C but not B primarily by rupture of the hexosaminidic bonds, the end products being tetrasaccharides[44] and the effect of our enzyme preparations might be due to degradation of polysaccharide of this type present in the matrix. It is unlikely that their effect is due to this alone, however, for one hyaluronidase preparation had almost no effect on the load extension curve. Like the others it was highly active against a preparation of chondroitin sulphate–protein complex but did not degrade this substance to the same extent.

It seems likely that the greater activity of the other hyaluronidase preparations is due to the presence of enzyme(s) other than hyaluronidase which, either alone or in conjunction with hyaluronidase, are responsible for damaging collagen fibres. The observation that those hyaluronidase preparations which damage the tendon fibres markedly reduce the viscosity of gelatin solutions suggests that they contain a proteolytic impurity. Only a limited proteolysis would be necessary to account for the observed change of viscosity and preliminary experiments[45] show that in fact no

extensive proteolysis occurs. The gelatins used were of a high degree of purity but the possibility cannot be excluded that the observed effects were due to degradation of mucoprotein impurity, present in very small amount but strongly bound to the gelatin[46].

The non collagenous component which appears to be at least partly responsible for the cohesion of tendon fibres is thus something other than chondroitin sulphate A or C or hyaluronic acid and is probably protein. DAY[29,30] and HARKNESS AND HARKNESS[31] found that the mechanical stability of loose connective tissue of rat and mouse and of rat uterus was markedly reduced when the tissues were treated with trypsin but not when they were treated with testicular hyaluronidase (Bengers, ovine). HARKNESS AND HARKNESS[31] concluded that non-collagenous protein contributes significantly to the mechanical stability of the collagen frame work of the rat uterus. In view of the work of HODGE *et al.*[42] suggesting that trypsin has a limited proteolytic action on collagen itself in solution, HARKNESS[2] has expressed doubt as to the validity of this conclusion but the more recent observations of KÜHN *et al.*[47] suggest that the site of trypsin attack resides in non collagenous protein present as an impurity in collagen preparations. Thus the stabilising function of non collagenous protein which we postulate for rat tail tendon fibres may be of more general occurrence. This protein may be visualised as a macromolecular cross-link between fibres and/or between fibrils. It may be of considerable length and flexibility and its deformation could contribute appreciably to fibre extension.

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