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Chondroitin Sulphuric Acid as a Factor in the Stability of Tendon

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The intercellular substances of connective tissue consist of fibrillar and amorphous components. On morphological grounds the histologists have postulated the presence of an amorphous substance cementing collagen fibrils together into fibre bundles, and an amorphous ground substance in which these bundles are embedded (Maximow & Bloom, 1948). Meyer and his co-workers (Meyer & Smyth, 1937; Meyer & Chaffee, 1941) have isolated the mucopolysaccharides chondroitin sulphuric acid (CSA) and hyaluronic acid from skin, cartilage and tendon, and these substances have been identified with the cement and ground substances, respectively. However, apart from the work of Meyer, Palmer & Smyth (1937) on protein-CSA complexes, of Partridge (1948) on cartilage and of Einbinder & Schubert (1951) on the binding of CSA by collagen, little information is available concerning the association of the mucopolysaccharides and collagen.

Rat-tail tendon can be dissolved in dilute acetic acid (Nageotte, 1927), and from the solution fibres can be reconstituted by dialysis and salt precipitation (Huzella, 1932; v. Buzagh, 1942), and by addition of mucopolysaccharides (Highberger, Gross & Schmitt, 1951). When examined under the electron microscope fibrils precipitated with 0.7–1.5% (w/v) potassium chloride showed the charac-

teristic 640 Å spacing (Bahr, 1950). At these concentrations, needle-shaped crystals or tactoids were produced, having the striated structure of collagen, while outside this range (0.5–10%) long, non-striated, fibrils were formed (Vanamee & Porter, 1951). Mucopolysaccharide-precipitated fibrils were found to have a wide spacing of up to 2000 Å, although small concentrations of serum mucoprotein gave the 640 Å spacing (Highberger *et al.* 1951). This was also shown for heparin-precipitated fibrils (Morrione, 1952). However, CSA-precipitated fibrils were either non-striated or had spacings up to 2000 Å (Highberger *et al.* 1951).

In these studies little emphasis was placed on obtaining mucopolysaccharide-free collagen, the tissue being usually extracted with 10% (w/v) sodium chloride or 30% potassium chloride. Partridge (1948) and Woodin (1952) showed that extraction with 10% (w/v) calcium chloride was necessary to remove all the mucopolysaccharide, and this method involved the solution of protein that appears to be of collagenous origin.

Before studying the relationship of mucopolysaccharide to collagen, it seemed essential to obtain mucopolysaccharide-free collagen by other methods. It has been reported (Meyer, Chaffee, Hobby & Dawson, 1941; Madinaveitia & Stacey, 1944) that

testicular hyaluronidase is active against CSA, the mucopolysaccharide component of tendon. It is probable that the same enzyme is active against both hyaluronic acid and CSA (Meyer *et al.* 1941; Humphrey, 1946), but only CSA occurs in tendon. Incubation of tendon with hyaluronidase caused gross changes which were visible to the naked eye to occur, the tendon becoming swollen and frayed, splitting up longitudinally into smaller units. It was decided, therefore, to study this phenomenon in detail. A preliminary communication on part of this work has already been made (Jackson, 1952).

EXPERIMENTAL

Material and methods

Tendon preparation. Fresh calf tendo Achillis was freed from gross fat and loose connective tissue, minced and then cut up finely with scissors, defatted with ethanol and ether, washed with 10% (w/v) NaCl and distilled water, dehydrated in acetone and dried *in vacuo*.

Enzyme preparation. Crude testicular hyaluronidase was prepared by the method of Madinaveitia (1941). This preparation was tested against CSA extracted from bovine trachea (Einbinder & Schubert, 1950) by measuring the liberation of reducing sugars. The relative activity of this preparation compared with that of a standard was not determined.

Shrinkage temperature. This was carried out by placing single fibres in water in a boiling tube fitted with a thermometer. The boiling tube was placed in a water bath, the temperature raised slowly and shrinkage temperature noted.

Total nitrogen. This was determined by the micro-Kjeldahl technique using the distillation apparatus of Markham (1942), digestion being carried out with SeO_2 as catalyst.

Dissolution of tendon

Treatment with hyaluronidase. Tendon (1 g./100 ml. of buffer) was incubated at 37° with hyaluronidase (2 mg./ml.) in 0.1 M-sodium acetate buffer at pH 5.2 containing 0.15 M-NaCl (A) (Meyer, 1951). A further portion of tendon was incubated in the same solution with the enzyme omitted (B). The material was incubated for 72 hr., incubating solutions being changed every 24 hr. The tendon was filtered off, washed with saline and distilled water, dehydrated with acetone and dried *in vacuo*.

Dissolution by acetic acid. A 1 g. sample of each of A and B was shaken with 100 ml. 0.4% (v/v) acetic acid for 24 hr., centrifuged, and the supernatant decanted. The extraction was repeated six times, the extracts being collected separately. The residue was washed and dried as before and weighed. Total N was determined on each extract.

Dissolution of reconstituted fibres

Fibres were reconstituted from a solution of enzyme-treated tendon by two methods: (1) by addition of an equal volume of 10% (w/v) NaCl (Huzella, 1932); (2) by addition of an equal volume of CSA (1 mg./ml.) (Highberger *et al.* 1951).

A portion of the CSA-precipitated material was incubated with hyaluronidase as described previously. All three preparations were washed in saline and water, dehydrated

with acetone and dried *in vacuo*. A sample (100 mg.) of each preparation was weighed and shaken with 50 ml. 0.4% (v/v) acetic acid for 24 hr., centrifuged, washed and dried as before and reweighed.

RESULTS

Dissolution of tendon after hyaluronidase treatment

During enzyme treatment the tendon fibres became swollen and frayed, and split up longitudinally into smaller units. During the first extraction A became considerably more swollen than B. The results (Table 1) show that after pretreatment of tendon with hyaluronidase 85% was soluble in dilute acetic acid as compared with 25% of the untreated tendon.

Table 1. *Effect of pre-treatment with hyaluronidase on the extraction of tendon by 0.4% (v/v) acetic acid*

(Tendon (1 g.) incubated with 100 ml. of 0.1 M-sodium acetate buffer, pH 5.2, containing 0.15 M-NaCl. Enzyme concentration 1 mg./ml. The figures represent average values of three experiments.)

Tendon	Initial weight (g.)	Residue weight (g.)	Dissolved (% initial weight)
Untreated	1.026	0.769	25.2
Enzyme-treated	1.016	0.153	84.9

Fig. 1. Total nitrogen of 0.4% (v/v) acetic acid extracts of (a) native tendon, (b) tendon treated with hyaluronidase.

Determination of total nitrogen of the extracts (Fig. 1) showed that most of it was made soluble during the first two incubations. The low nitrogen content of the first extract of A was due to the considerable swelling which took place, 85 ml. of the extracting solution being imbibed by the tendon.

Dissolution of reconstituted fibres

The results (Table 2) show that almost the whole of the salt-precipitated collagen and of the enzyme-treated CSA-precipitated collagen was redissolved by 0.4% (v/v) acetic acid, while CSA-precipitated collagen was virtually insoluble.

Table 2. *Dissolution by 0.4 % (v/v) acetic acid of fibres reconstituted from solution in 0.4 % (v/v) acetic acid by different treatments*

(Samples (100 mg.) shaken with 50 ml. of acetic acid. Fibres in Exp. 3 incubated with 50 ml. of acetate buffer, pH 5.2, containing 0.15M-NaCl. Enzyme concentration 1 mg./ml. The figures represent average values of three experiments.)

Exp. no.	Type of reconstitution of fibre	Initial weight (mg.)	Residue weight (mg.)	Dissolved (% initial weight)
1	Salt precipitation	103	5	95.1
2	CSA precipitation	110	106	2.7
3	CSA precipitation followed by incubation with hyaluronidase	105	1	99.0

Shrinkage temperature

Native tendon shrinks at 67°. On enzyme treatment the shrinkage temperature was found to be 53°. It was also observed that removal of CSA from CSA-precipitated fibres reduced the shrinkage temperature from 55 to 47°. The lowest shrinkage temperature found was that of salt-precipitated fibres (43°). The results (Table 3) show that absence of CSA or its degradation by hyaluronidase, depresses the temperature at which collagenous fibres shrink.

Table 3. *Shrinkage temperature of various fibre preparations*

(Preparations 2 and 5 treated with hyaluronidase in 0.1M-sodium acetate buffer, pH 5.2, containing 0.15M-NaCl. Enzyme concentration 1 mg./ml. Results are the average of six determinations.)

Exp. no.	Type of fibre	Shrinkage temperature (°)
1	Native	67
2	Enzyme-treated	53
3	Salt-precipitated	43
4	CSA-precipitated	55
5	CSA-precipitated and then enzyme-treated	47

DISCUSSION

The swelling of collagenous material such as tendon and skin is considered to be the result of the excess osmotic pressure (swelling pressure) in the protein phase, arising from the difference in concentration of diffusible ions in the protein and external phase, equilibrium being reached when the osmotic pressure is balanced by the cohesive forces of the protein (Procter & Wilson, 1916).

The enzyme treatment of tendon was carried out at pH 5.2. At this pH minimum swelling would be expected; since the swelling pressure is the same in each case, and the enzyme-treated tendon (A) was more swollen than the untreated tendon (B), it appears that enzyme treatment has decreased the cohesive forces. This increased swelling of A was also noted during the first extraction with 0.4 % (v/v) acetic acid. The increased solubility of tendon

in dilute acetic acid after enzyme treatment suggests that the swelling pressure has overcome the cohesive forces, causing breakdown of tendon structure. This breakdown of structure was only partial with untreated tendon, but was almost complete with the enzyme-treated material, presumably, because this treatment, as suggested above, has greatly reduced the cohesive forces. The results of the experiments with reconstituted fibres lead to the same conclusion, since absence or removal of CSA resulted in almost complete re-solution.

The shrinkage temperature has been shown to be a measure of the structural stability of collagen expressed in temperature units (Theis, 1946). Astbury (1940) has suggested that any interference with the interaction of any inter-chain linkage must inevitably influence the shrinkage temperature. If, therefore, CSA is concerned with inter-chain linkages, removal of CSA from tendon or its absence from reconstituted fibres should result in a reduction of the shrinkage temperature. This was in fact found, and provides further evidence for the thesis that CSA is responsible to some extent for the structural stability of collagenous fibres.

Strong alkali and calcium chloride solutions have been widely used for the extraction of mucopolysaccharides such as CSA (Meyer & Smyth, 1937). Hence treatment with these reagents would, on the evidence presented here, be expected to reduce the stability of collagenous tissues. Partridge (1948) has pointed out that solvents which are good extractors of CSA from cartilage also reduced the shrinkage temperature of rat-tail tendon. Theis (1946) showed that the presence of 2M-sodium chloride in the external phase reduces the effect of pH on swelling and shrinkage temperature to a minimum except at a pH greater than 10 when swelling is still very prominent and shrinkage temperature falls rapidly. In contrast, the presence of calcium chloride increases swelling and causes a considerable fall in shrinkage temperature. Bowes & Kenten (1950), in a careful study of the swelling of hide, conclude that the rapid increase in swelling above pH 10 is due to a decrease of cohesion, caused mainly by a breakdown of inter-molecular linkages. On the basis of the results of

Partridge (1948) they suggested that some of the intermolecular linkage may be due to the mucopolysaccharides present in hide. Indeed, all these experiments lead to the conclusion that conditions which are favourable to the extraction of mucopolysaccharide are associated with a striking reduction in the stability of the tissues studied. It could be argued (Theis, 1946) that this reduction in the very high pH range is due to a pronounced effect of alkali on hydrogen bond structures. This might lead to a concomitant release of bound mucopolysaccharide. However, it is not certain that strong alkali or calcium chloride does in fact break down the hydrogen bonds. Nor would the enzyme treatment described in this paper be expected to have any effect on these bonds; this treatment leads to a reduction in the stability of tendon similar to that demonstrated by the results of the workers discussed above. Thus an alternative interpretation is that CSA is in fact taking part in the intermolecular linking in the collagenous fibres of connective tissue and that its removal leads to a reduction of fibre stability. Partridge (1948) has suggested that CSA is cross-linked to collagen in cartilage, and a similar suggestion concerning corneal mucopolysaccharide has also been put forward (Woodin, 1952).

The results presented in this paper indicate that CSA plays an important part in the stability of tendon, but this tissue contains only a small amount of mucopolysaccharide. It is possible, therefore, that it enters into the structure of tendon at a fairly high level of aggregation of collagen molecules. There is no evidence to show how CSA is incorporated into the tissue structure, but it is probably firmly linked, since drastic chemical treatment is required to extract all the mucopolysaccharide from

such tissues as tendon, cartilage and cornea, and these conditions cause solution of protein of collagenous origin (Meyer *et al.* 1937; Partridge, 1948; Woodin, 1952). Considered in this way the structure of tendon can be visualized as bundles of polypeptide chains bound together by inter-chain forces, these bundles being further aggregated by CSA acting as a cement substance.

Tendon is a specialized form of connective tissue, and it may well be that in other connective tissue structures the relationship between collagen and mucopolysaccharide is different. Nevertheless, the evidence presented in this paper underlines the possible importance of mucopolysaccharides for the stability of connective tissue, and indicates the desirability of carefully considering this factor in studies of the pathogenesis of the so-called collagen diseases in which breakdown of connective tissue bundles is a prominent feature.

SUMMARY

1. Treatment of tendon by testicular hyaluronidase was found to lead to a decrease in the stability of tendon as measured by an increase in swelling, by increased solubility in dilute acetic acid and a decrease in shrinkage temperature.

2. Similar experiments with reconstituted collagen produced the same results.

3. On the basis of this evidence and that of other workers, it is concluded that the mucopolysaccharides play an important part in stabilizing the collagenous fibres of connective tissue.

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