The Nature of Collagen-Chondroitin Sulphate Linkages in Tendon

BY D. S. JACKSON
Rheumatism Research Centre, University of Manchester

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In previous communications it has been suggested that chondroitin sulphate plays an important part in stabilizing the structure of tendon (Jackson, 1953; Jackson & Ball, 1953). What little evidence is available suggests that the mucopolysaccharide is firmly linked to the collagen of tendon (Meyer, 1952). Einbinder & Schubert (1951) found that the uptake of chondroitin sulphate by purified tendon collagen was maximal at pH 3 and minimal in the physiological pH range, and on this evidence they suggested that the linkage is not salt-like. Mörner (1889) held that chondroitin sulphate occurred in cartilage as an alkali-metal salt, while Meyer, Palmer & Smyth (1937) were of the view that cartilage contains a salt of chondroitin sulphate and collagen. Partridge (1948) favours an explanation half-way between these two extremes, and considers that the strongly acidic sulphate groups are held in combination with some of the basic protein residues, the net charge of the complex being adjusted mainly by competition between alkali-metal ions and hydrogen ions for carboxylic acid residues in both protein and polysaccharide. Woodin (1952) considers that in cornea the collagen-mucopolysaccharide linkages are similar to those between adjacent collagen polypeptide chains.

Mammalian collagen is generally considered to be stabilized by (1) electrovalencies located at polar side chains, forming salt-like cross-linkages, and (2) co-ordinate valencies between adjacent peptide groups (hydrogen bonds) (Gustavson, 1942a).

Astbury (1940) has stated that anything which interferes with the interaction of any interchain linkage must inevitably influence the thermal transformation temperature. Their (1946) concludes that the shrinkage temperature may be defined as the specific point at which disruptive tendencies exceed the cohesive forces, thus making this temperature an actual measurement of the structural stability of collagen expressed in temperature units.

Further, it has been pointed out that an evaluation of the relative importance of the two types of directed valency present in tendon, can be obtained by measuring the shrinkage temperature of collagen after treatment specific for these two linkages (Gustavson, 1949a). β-Naphthalene-sulphonic acid will inactivate the basic groups completely, eliminating the salt-like cross-linkages, without swelling the collagen and without affecting the co-ordinative activity of the peptide groups (Gustavson, 1942a). Using this reagent he has shown that the shrinkage temperature of skin is reduced by 10–12°, whereas urea, generally expected to break hydrogen bonds, lowers this temperature by about 40°. This means that hydrogen-bond formation is the more important in determining fibre stability. Strong solutions of calcium chloride have the same effect as urea (Theis, 1948; Partridge, 1948), dilute solutions also effecting some reduction in shrinkage temperature (Theis, 1940).

The effect of concentrated calcium chloride solutions is considered to be due to an interaction of the neutral salt with hydrogen bonds, causing a weakening and rupture of these stabilizing links (Gustavson, 1926), while an ionic interaction probably predominates in dilute solutions (Gustavson, 1949a). For these reasons shrinkage temperatures were determined in a wide range of calcium chloride concentrations (0–2-7 x), as a measure of the remaining linkages after treatment with the various reagents used.

Periodic acid oxidizes 1:2 glycol and 1-amino-2-hydroxy groups with the formation of aldehyde groups and was introduced as a method of splitting carbon-carbon bonds by Malaprade (1928, 1934). A preliminary experiment showed that periodic acid oxidation was effective with chondroitin sulphate. If the hydroxyl groups of chondroitin sulphate are concerned with hydrogen-bond formation in tendon, periodic acid oxidation of the hydroxyl groups should lead to a reduction in shrinkage temperature.

In this communication the results of applying these methods to rat-tail tendon and reconstituted collagen are reported. The results obtained have been interpreted as confirming previous suggestions concerning the role of chondroitin sulphate in stabilizing tendon (Jackson, 1953; Jackson & Ball, 1953) and as pointing to both salt-like cross-linkages and to hydrogen bonding as the forces concerned in the collagen-chondroitin sulphate association. The presence in tendon of a further stabilizing mucoprotein is also suggested.

EXPERIMENTAL METHODS

Materials

Tendon was obtained from the tails of freshly killed rats, cut into 1 cm. lengths and stored in the frozen state. Care was taken not to damage the fibres, any damaged ones being rejected.
Reconstituted collagen was prepared by precipitation from a solution of calf tendon Achilles in 0-4 M acetic acid, with 10% (w/v) NaCl and with chondroitin sulphate solution (1 mg/ml) as previously described (Jackson, 1953). The precipitate consisted of a loose network of fine fibrils of varying dimensions, which made it difficult to obtain samples of uniform size. However, samples approximately 1 cm. in length were used for the shrinkage temperature determinations. Salt-precipitated collagen will be referred to as s.p.c., collagen precipitated by chondroitin sulphate as c.p.c.

Treatment of materials

Hyaluronidase. The method used was that described in the previous communication (Jackson, 1953).

β-Naphthalenesulphonic acid. Samples of 0-5 g. of each preparation were soaked in 5% (w/v) β-naphthalenesulphonic acid (pH 1-2) for 24 hr., and washed thoroughly with distilled water. The excess of acid was titrated with 0.2N-NaOH using methyl orange as indicator; the amount of acid fixed was determined by difference. 0.86–0.90 m-equiv. of acid were fixed by all the preparations. Microscopic examination of the tendons after treatment revealed no evidence of swelling, although similar treatment with HCl at the same pH (1-2) caused considerable swelling and destruction of the fibre structure.

Periodic acid. The reagent was prepared by dissolving 0-5 g. NaIO4, 3H2O and 0-5 ml. 70% (v/v) HNO3 in 100 ml. water. This solution has a pH of 2.5, and in order to suppress the swelling of collagen which would occur at this pH, NaCl was added to a concentration of 2M. The material was incubated in this solution at 25° for 24 hr. Similar reaction mixtures, with HCl at pH 2-5 in place of the periodic acid, were set up as controls. After treatment, all materials were washed thoroughly with distilled water.

Shrinkage temperature determinations

These were carried out in the apparatus previously described (Jackson, 1953). After being subjected to the appropriate treatment, the shrinkage temperature determinations were carried out in triplicate in increasing concentrations of CaCl2 (0-2-7 M). The temperature was increased rapidly up to 5° below the shrinkage temperature and then very slowly in steps of 1° and held at each step for 2 min. Little or no swelling was apparent during the determinations but the specimen became translucent at about 8-10° below the actual shrinkage temperature. Preliminary experiments with native tendon and s.p.c. indicated that no pH change occurred in the media during the shrinkage temperature determinations. Preliminary determinations made on twenty specimens of s.p.c. and of tendon gave, for s.p.c. 42 ± (s.d. 1°); for tendon 66 ± (s.d. 1°). Shrinkage temperature was plotted against concentration of CaCl2.

RESULTS

Untreated material. The results are shown in Fig. 1. The curve for native tendon is similar to that obtained by Theis (1946). There was first a rapid fall in shrinkage temperature with CaCl2 concentrations up to 0-3 M, followed by a slower fall. Collapse of the collagen structure at room temperature occurred at 2.4 M-CaCl2. Treatment of tendon with hyaluronidase reduced the shrinkage temperature in water by 12° to 55°. The shrinkage temperature remained at this level up to 0.8 M-CaCl2, so that there was no initial rapid fall. The shrinkage temperature of enzyme-treated tendon continued higher than that of untreated tendon from 0.1 M to 1.5 M-CaCl2. The curve of c.p.c. followed that of untreated tendon at a lower level and, similarly, the curve of s.p.c. was parallel to that of enzyme-treated tendon; the fall in shrinkage temperature of s.p.c. was more rapid than that of the other three preparations, complete structural collapse at room temperature occurring at 1.5 M-CaCl2 as compared to 2.1–2.4 M.

![Fig. 1. Shrinkage temperature in CaCl2.](image)

![Fig. 2. Shrinkage temperature in CaCl2 after treatment with 5% β-naphthalenesulphonic acid.](image)
After treatment with β-naphthalene sulphonphonic acid.
The results are shown in Fig. 2. The decrease of 10° in the shrinkage temperature of native tendon in water was similar to that reported for skin collagen by Gustavson (1942a). The initial rapid fall in shrinkage temperature shown by untreated tendon was reduced by this treatment from 14° to about 8°. There was also a more rapid fall from 0·3 M-CaCl₂ onwards, room-temperature shrinkage occurring at 1·5 M-CaCl₂ after treatment, compared with 2·4 M-CaCl₂ before treatment. The shrinkage temperature in water of hyaluronidase-treated tendon was reduced by a further 4° (from 55 to 51°).

After treatment, s.p.c. formed a sticky, glue-like mass and became extremely swollen during washing with distilled water and disintegrated rapidly. The material dissolved in 0·9% (w/v) NaCl, and increasing the salt concentration to 5% (w/v) NaCl caused a precipitate to appear which was found to be fibrous. C.p.c. also formed a glue-like mass, which, on washing with water, disintegrated to a fine suspension but was insoluble in 0·9% (w/v) NaCl. Increasing the salt concentration to 5% (w/v) NaCl caused the suspension to coagulate into a granular precipitate in which no fibrous structure was visible under the optical microscope.

After treatment with β-naphthalenesulphonic acid, s.p.c. collapsed at room temperature in the most dilute calcium chloride, c.p.c. showing room-temperature shrinkage at 0·3 M-CaCl₂.

After periodic acid treatment. The results are shown in Fig. 3. The effect of periodic acid treatment was quite striking. The shrinkage temperature of tendon in water was reduced by 24°, while that of hyaluronidase-treated tendon, which started some 12° lower, was reduced by 10–12°, so that the curves for enzyme-treated and native tendon start at the same level and are identical throughout. The initial rapid fall in shrinkage temperature was replaced by a slight rise, with a maximum at 0·4 M-CaCl₂, as compared with 0·2 M for s.p.c. Room temperature shrinkage of tendon occurred at 2·1 M-CaCl₂ as compared with 2·4 M-CaCl₂ before periodic acid treatment. This treatment had no effect on s.p.c., the two curves being identical before and after treatment, but treatment with periodic acid reduced the shrinkage temperature of c.p.c. in water to that of s.p.c., the two curves now being identical.

It was also noticed that tendon became considerably more swollen in water after treatment and split up into a mass of very fine fibrils.

Treatment of tendon with β-naphthalene sulphonphonic acid following periodic acid oxidation caused a further reduction of shrinkage temperature in water from 42 to 37°.

DISCUSSION

In calcium chloride, the shrinkage temperature curves of native tendon and c.p.c. are parallel, as are those of hyaluronidase-treated tendon and s.p.c. The essential difference between these two groups of curves lies mainly in the absence of an initial rapid fall of the shrinkage temperature at lower concentrations of CaCl₂ (0–0·3 M) (Fig. 1), in the latter group. These observations suggest that the first part of the shrinkage temperature curve of tendon relates to the chondroitin-collagen linkages. Theis (1946) interpreted this initial fall in shrinkage temperature in dilute calcium chloride as being due to the effect on ionic links between adjacent collagen polypeptide chains.

In view of the absence of swelling after treatment with β-naphthalenesulphonic acid, and the unchanged pH of the media during shrinkage temperature determinations, it seems likely that the hydrogen bonds between adjacent collagen-polypeptide chains remain unaffected and that the effect of this reagent is confined to the abolition of ionic bonds, as has been suggested by Gustavson (1942a). If this is so, then from Theis's interpretation, the initial fall in shrinkage temperature should not occur after treatment with β-naphthalenesulphonic acid, whereas this treatment reduces the initial fall by only 6° (from 14 to 8°) (Fig. 2). Moreover, the curve for s.p.c., which has been shown to contain salt-like cross-linkages (see below), fails to show the expected fall in shrinkage temperature in
dilute calcium chloride (Fig. 1). Thus the initial fall in shrinkage temperature may not be a function of intercollagen cross-links. In view of the effect of hyaluronidase on the shrinkage temperature curve for tendon, and of the absence of chondroitin sulphate in reconstituted collagen, an alternative interpretation is that the effect of low concentrations of calcium chloride on the shrinkage temperature is due to the splitting of chondroitin sulphate-collagen links, part of which are ionic, the remainder presumably being hydrogen bonds. This hydrogen bonding might take place through the hydroxyl groups of chondroitin sulphate and treatment with periodic acid, which would be expected to destroy these groups (Malaprade, 1928), does in fact abolish the initial fall in shrinkage temperature (Fig. 3). In this respect periodic acid is even more effective than hyaluronidase, the shrinkage temperature in water being reduced by 24° as compared with 10–12°. Periodic acid will oxidize the free hydroxyl-amino acids such as serine and threonine; but as the amino groups of these amino acids are involved in peptide links in proteins, it is unlikely that these amino acid residues in collagen polypeptide chains will be oxidized. Moreover, the shrinkage temperature curve of S.P.C. is unaffected by periodic acid treatment. The results with periodic acid on tendon may mean (a) that it is more destructive than hyaluronidase, or (b) that some other unsuspected stabilizing mucopolysaccharide or mucoprotein, unaffected by the enzyme, is present in tendon. Further study of the composition of tendon would be necessary before any decision could be made between these alternative explanations.

Treatment of native tendon with β-naphthalenesulphonic acid reduces its shrinkage temperature in water by 10°, whereas after hyaluronidase and periodic acid treatment, the same reagent produces a reduction in shrinkage temperature of only 4°. A possible interpretation of these facts would be that only part of the 10° reduction in shrinkage temperature is due to destruction by β-naphthalenesulphonic acid of ionic links between adjacent collagen chains, which would probably remain unaffected by hyaluronidase and periodic acid, and that these reagents affect the ionic links between mucopolysaccharide and collagen.

Complete structural collapse at room temperature of both hyaluronidase-treated and native tendon, occurs only in concentrated calcium chloride solutions, which indicates that the more important bonds stabilizing tendon are hydrogen bonds between adjacent collagen-polypeptide chains.

After periodic acid oxidation, room-temperature shrinkage occurs in 2·1 M-CaCl₂ as compared with 2·4 M-CaCl₂ for untreated tendon, which suggests again that bonds between adjacent collagen chains are not affected.

Some light is also thrown on the structure of salt-precipitated collagen by the effect of β-naphthalenesulphonic acid treatment. The complete structural collapse at room temperature in dilute calcium chloride, its solubility in 0·9% NaCl, and the swelling and disintegration in water all suggest that the stabilization of its structure is mainly by salt links, hydrogen bonds playing a very small part. Its behaviour is very similar to that of fish-skin collagen, which has a shrinkage temperature of 42° (Gustavson, 1942b) and forms a glue-like mass with α-lignosulphonic acid, which has a similar effect to β-naphthalenesulphonic acid (Gustavson, 1949b). Gustavson has interpreted this evidence as meaning that salt-like links mainly hold the structure together, the major proportion of the peptide groups not being internally compensated.

The observation that the salt-reconstituted collagen used in this work is soluble in 0·9% (w/v) NaCl, after treatment with β-naphthalenesulphonic acid, and can be reprecipitated from this solution in a fibrous form by increasing the salt concentration to 5% (w/v) NaCl, is interesting in view of the similar behaviour of collagen reconstituted from a collagen solution in 1·2% acetic acid, without previous treatment with β-naphthalenesulphonic acid (Jackson & Ball, 1953). This supports the suggestion then made that the end-to-end links in this material must be very weak, and suggests that these links involve only van der Waal’s forces, since salt links are eliminated by β-naphthalenesulphonic acid.

The behaviour of C.F.C. after this treatment with β-naphthalenesulphonic acid provides further evidence of the function of chondroitin sulphate as a stabilizing factor.

In conclusion, if we accept Gustavson’s suggestion that the effect on shrinkage temperature of various specific reagents may be used as a method of evaluating the relative importance of the two types of directed valency (Gustavson, 1949a), four-fifths of the stabilizing bonds in tendon are probably hydrogen bonds, the remainder being ionic cross-links. The linkages between chondroitin sulphate and collagen are probably of both types, with ionic links less important than the hydrogen bonding, together accounting for about a quarter of the total stabilizing bonds.

**SUMMARY**

1. The shrinkage temperature curves in calcium chloride of native rat-tail tendon, hyaluronidase-treated tendon, salt-precipitated and chondroitin sulphate-precipitated collagen have been determined before and after treatment with hyaluronidase, with β-naphthalenesulphonic acid and with periodic acid.
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2. Chondroitin sulphate was found to play a part in the stabilization of tendon. It is suggested that a quarter of the stabilizing linkages are between chondroitin sulphate and collagen, consisting of 40% salt-like links and 60% hydrogen bonds.

3. Some other mucopolysaccharide or mucoprotein may also be concerned in this stabilization.

4. Salt-precipitated collagen is similar to fish skin in being stabilized mainly by salt-like cross-links.

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REFERENCES


Chromatography of Pyruvic Acid and of Acetoacetic Acid as their 2:4-Dinitrophenylhydrazones

By S. MARKEES
Medical Laboratories of F. Hoffmann-La Roche and Co. Ltd., Basle (Switzerland)

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The problem of the separation and the quantitative estimation of keto acids in the blood is of the greatest interest on account of the importance of these metabolic intermediates. Markees & Gey (1953) dealt with the quantitative paper chromatography of pyruvic acid as the 2:4-dinitrophenylhydrazone and showed that the derivatives of this \( \alpha \)-keto acid form two sharply distinct spots with the \( R_P \) values of 0-36 (A) and 0-57 (B). This chromatographic separation has already been observed and described by other authors (Cavallini, Frontali & Toschi, 1949; LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Hockenhull, Hunter & Herbert, 1953; Stewart, 1953). The formation of these two spots A and B is most probably due to the formation of cis and trans isomers.

The publication of El Hawary & Thompson (1953), in which the 2:4-dinitrophenylhydrazones of \( \alpha \)-oxoglutaric acid, oxaloacetic acid, pyruvic acid, acetoacetic acid and acetone have been investigated chromatographically, prompted us to a renewed investigation of this problem. They found on analysis of their chromatograms a spot in the \( R_P \) region of 0-46–0-64 which they believed to be due to acetoacetic acid. Experimentally they proceeded in a very similar manner to ourselves, with the difference that we used ethyl acetate as a solvent for adding the substances to the chromatogram, while they, instead, employed sodium hydroxide plus phosphate buffer. The developing solvent was identical in both cases (butanol:ammonia:water) except that they added 10% (v/v) ethanol which we did not. The question now arises whether the spot in the \( R_P \) region of 0-46–0-64 can be considered as due to the 2:4-dinitrophenylhydrazone of acetoacetic acid, as has been suggested by El Hawary & Thompson (1953), or whether it has possibly been mistaken by them for the spot which we call B, and which appears to be due to a reaction product of 2:4-dinitrophenylhydrazine with pyruvic acid.

EXPERIMENTAL AND RESULTS

In order to clear up this point we spotted the chemically prepared 2:4-dinitrophenylhydrazone of acetoacetic acid directly, and we also carried out an analysis of blood to which acetoacetic acid had been added. In one case we also boiled the blood filtrate before the addition of 2:4-dinitrophenylhydrazine, and, in another case, we analysed it without boiling. The experimental details were exactly the same as described in our earlier work (Markees & Gey, 1953). Controls using buffered sodium hydroxide as the solvent did not show any change in the \( R_P \) values of the various 2:4-dinitrophenylhydrazones. The results of these experiments can be summarized as follows:

Chemically prepared acetoacetic acid 2:4-dinitrophenylhydrazone gives a spot with an \( R_P \) value of 0-48. We also find a spot with the \( R_P \) value of 0-94 which corresponds to the acetone hydrazine formed by decomposition.

Chemically prepared pyruvic acid 2:4-dinitrophenylhydrazone always forms the above-mentioned spots A and B. The \( R_P \) value of 0-57 of B is quite different from that of the dinitrophenylhydrazine of acetoacetic acid.