EFFECT OF CYCLIC EXTENSION ON THE PHYSICAL PROPERTIES OF TENDON COLLAGEN AND ITS POSSIBLE RELATION TO BIOLOGICAL AGEING OF COLLAGEN

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So far as we are aware no attention has been directed to the effect of cyclic extensions on the mechanical and thermal properties of tendon collagen, although investigations with static extension have been numerous. In general, static extensions in saline of less than 2 per cent held for periods of 20 h or more at room temperature produce structural changes such that the sample will begin to shrink between 50°C and 60°C, rather than at its normal shrinkage temperature, 60°C. However, these changes are recoverable if the sample is allowed to rest in a slack condition for 10 h. For extensions greater than 3 per cent the structural changes are not recoverable and in this event the shrinkage temperature may be lowered to the vicinity of 40°C after times of extension of 70 h or more. Extensions greater than 2–3 per cent also give rise to irreversible damage so far as mechanical properties alone are concerned, for example, the Young's modulus is reduced.

While the work recorded here has been undertaken for its own intrinsic interest, it is worth noting that cyclic extension of tendon is a more realistic experiment than the static one from the point of view of physiology. To first approximation, tendon operates under cyclic conditions in the in vivo state.

Rat-tail tendon, usually from rats aged 3–4 months, was used and the specimens were tested in 0.9 per cent saline solution at 20°C. The extension was limited to less than 2 per cent, which is the region in which mechanical properties are reproducible. An 'Instron' extensometer was used in the experiments, and with this instrument a number of different cycling conditions are possible. We have used the predetermined limits: (1) zero extension–given extension; (2) zero extension–given load; (3) given load–given load. In the first two cases the period was about 175 min, while in load–load cycling the period was less. The main difference between conditions (1) and (2), and (3), is that in (3) the sample has no period of rest at zero load. As well as examining the effect of cycling on the load-extension curve, any changes in the X-ray diagram and in the thermal stability (as measured by the shrinkage temperature, T_s) were observed. This was done by halving a tendon, one-half being cycled. Both portions were then either X-rayed or heated for a T_s determination. Only high-angle X-ray pictures were taken, although some low-angle reflexions were discernible along the meridian. Pictures were obtained of both air-dried and wet samples using copper radiation nickel filtered. The air-dried samples were dried under zero tension after being washed free of sodium chloride. The other samples were mounted wet under zero tension in a sealed specimen holder in which an atmosphere of 100 per cent relative humidity was maintained. Shrinkage temperatures were measured by a force-temperature method. In this the sample is held at constant length (almost zero tension) while being heated at a constant
here it is seen that there is an initial weakening of the tendon for about the first 50 cycles and a consequent increase of $F_{\text{max}}$. A simultaneous small increase in length occurs which becomes noticeable after 200–300 cycles. Fig. 2(b) shows that after an initial set of cycles, in this case 150 (full line), the force in the fibre continues to increase (dotted line) even in the absence of cycling.

Fig. 3 shows an experiment in which the sample has been cycled between zero extension and a given fixed load. In this case the maximum extension in the first cycle was between 0-5 and 1 per cent, showing that the stiffening phenomenon occurs at the very beginning of the linear, reversible region of the sample’s load-extension curve. In all cases, complete recovery of the mechanical properties of the sample is only possible if the sample is rested in a slack condition for about 10 min between extensions.

The essential result is that the tensile stiffness of the tendon is increased.

**X-ray Diffraction Effects.** High-angle pictures for both wet and dry samples are shown in Figs. 4 and 5. As stated earlier, they have been taken on the two halves of one sample, a half of which was cycled. The cycled portions were photographed as soon as possible after the completion of cycling. In both the wet and dry samples the improvement in orientation in the cycled half is clear, for every reflexion. For the wet sample the improvement in the low-angle reflexion along the fibre axis is very prominent.

**Shrinkage Temperature.** The shrinkage temperatures were measured in 0.9 per cent sodium chloride as soon as possible after cycling. In every case the half which had been cycled contracted at a higher temperature and in most cases developed a greater force during contraction than the uncycled portion. Behaviour for one sample is shown in Fig. 6.

It is apparent from the foregoing account that while a small number of cyclic extensions (~ 50) of a tendon result in a weakening of the structure, prolonged cycling gives rise to a more oriented structure. This is shown most directly by the high-angle X-ray diffraction patterns and indirectly by the increase both in the slope of the load-extension curve and in the thermal stability.

Since in the *in vivo* state tendon operates under conditions which to a first approximation are cyclic, it is of interest to consider the possible relevance of the work just described to information already known concerning the dependence of the mechanical and thermal properties of collagen on biological age, that is, with cycling.

Some of the more obvious of these effects include: reduction in swelling*1, extraction*1, and an increase in shrinkage temperature*1, 13. Van Meeteren*14, 15 has shown that the shrinkage temperature increases with age, nevertheless finds a good correlation between the maximum force developed by a rat-tail tendon and increasing age.

Another type of evidence is that of the X-ray diagram. In this respect, Kratky et al.*16 have shown that for human
Achilles tendon (1) the spread of the 32 Å arc on the meridian and the 11 Å equatorial arc both decrease with increasing age; and (2) the relative intensity of the 32 Å increases with respect to the 11 Å arc, again with increasing age. (The 32 Å arc is taken to be the twentieth order of the 640 Å axial repeat.) Feitlberg and Kaunitz obtained similar results with human chordae tendineae. These results show that with age the collagen crystalites become better oriented with respect to the tendon axis and that the structure giving rise to the low-angle pattern also becomes more regular.

It has been suggested by a number of authors that those and other ageing effects are due to an increase in the number of cross-links in collagen, and direct evidence for such an occurrence has been claimed. Better orientation of the structure could give rise to increased interaction especially by weak bonds such as hydrogen bonds and van der Waals interactions, but given sufficient time it is possible that even strong covalent bonds (ester bonds) could form by mechanical movement allowing potentially reactive groups to approach suitable positions. An analogous suggestion for collagen gels has been made by Gross.

The question as to the rate of turnover of collagen in the body is crucial to this discussion. The general conclusion by most workers is that collagen is relatively inert metabolically, particularly in skin and tendon. The fact that changes are found to occur with age is itself an indication that turnover must be slow, or that any replacement of parts of the structure have to fit into the altered structure as they find it.

Since our experiments give rise naturally to the findings listed here concerning X-ray diagrams and thermal shrinkage, it might be suggested that mechanical cycling in vivo is a possible factor in the production of ageing effects in tendon. The results of Brown and Constable are significant in this respect. They found that the temperature of the onset of human fetal collagen in saline is only about 54° C, and shows little or no change during the entire fetal life. It is only after birth, when the movement of collagenous tissues increases dramatically that the shrinkage temperature begins to rise. For the age groups 0–14 years and 45–88 years their values are 58° C and 61° C, respectively.

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SEROTONIN RECEPTORS: V, SELECTIVE DESTRUCTION BY NEURAMINIDASE PLUS EDTA AND REACTIVATION WITH TISSUE LIPIDS

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Earlier investigations in this series have indicated that the receptor for serotonin in animal tissues is a specific lipid. This lipid can be extracted from serotonin-susceptible cells by fat solvents and behaves during chemical fractionation as do the complex lipids such as sphingolipids or phospholipids. The mechanism of action of serotonin seems to be to bring about active transport of calcium ions through the cell membrane by means of a sequence of specific, reversible, and cyclic reactions with the receptor lipid.

We have been attempting to isolate the receptor lipid in pure form, and for this purpose have been using assays based on the ability of the lipid to bring about the transport of calcium ions in competition with serotonin in the absence of cell particles in a system of water, benzene and butanol. Because these assay systems have certain shortcomings, we have been attempting to find a simple bioassay procedure (which might be more direct and specific) to supplement them, and aid in the purification and identification of the hormonal receptor. In this article, evidence will be presented to show that the serotonin receptor can be destroyed selectively in isolated smooth muscles by the combined action of neuraminidase plus EDTA (ethylenediamine tetraacetate or versene), and that the receptor can be restored to the defective tissue in vitro by addition of lipids extracted from serotonin-susceptible cells.

When a serotonin-susceptible tissue such as a horn of a rat uterus or a strip of a rat stomach was treated briefly with minute amounts of purified neuraminidase in the presence of EDTA, and these reagents were washed off, the tissue were found to be insensitive to the contractile action of serotonin. Their responsiveness to other contraction-inducing substances, such as acetylcholine, bradykinin, or calcium ions, remained largely undiminished. For rat stomach these facts are illustrated by the results in Table 1. Note that the responsiveness to serotonin was reduced about 100,000-fold. The neuraminidase alone, even in very large amounts (400 mu units), had no response to serotonin, and, as was shown previously, neither did the EDTA alone. Highly purified neuraminidase, either from Clostridium perfringens or from Vibrio cholerae, was effective when used with EDTA.

The very small amounts of purified neuraminidase required for this specific inactivation of the serotonin

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