OXYGEN PARTICIPATION IN THE IN VIVO AND IN VITRO AGING OF COLLAGEN FIBRES

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Summary We have examined, as a function of oxygen concentration, the in vivo and in vitro aging rates of tendon from animal species with widely differing life-spans. The results suggest that concomitant with the genetically determined aldimine type cross-linking reactions which probably are complete by the time the animal has reached physiological maturity, there is an on-going oxygen-mediated system of reactions which also effectively cross-link the structure. These reactions appear to proceed with their own intrinsic rate dependent only upon oxygen concentration, and independent of the particular species involved. They may not be required by connective tissues, but are an unavoidable consequence of the presence of free oxygen in tissues with a low rate of turnover.

Introduction The physical, mechanical and chemical changes which take place in collagenous tissues with in vivo aging are well known (1). A graphic example is the decrease in solubility. It is now well established that similar changes take place in vitro for both native (2,3) and reconstituted (4) collagen fibres. These aging effects are processes of consolidation; the tissue takes on the characteristics of a system which has become progressively bound by intermolecular cross-links. While much is known about some aspects of the cross-link chemistry of collagen (see for exmaple references 5 and 6), the story is far from complete. It is generally agreed that only one enzymatic process is necessary to begin the formation of cross-links. This is the oxidative deamination of lysyl or hydroxylysyl residues. The resulting aldehydes are then believed to condense with reactive groups such as the ε-amino group of hydroxylysine in neighbouring molecules. However, it is not clear whether the number of cross-links initially laid down remains constant, and their reactivity changes with time, or whether the number of cross-links increases with

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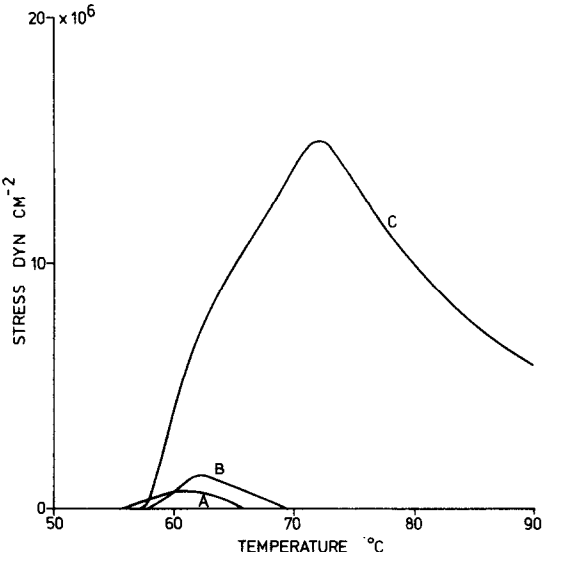


Fig.1. Isometric melting curves for rat tail tendon in 0.9% Nacl from an animal killed at 40 days.

A. No in vitro aging
B. 180 days in vitro aging in 0.9% Nacl containing 60 n mol ml^{-1} oxygen, 20°C.
C. 180 days in vitro aging in 0.9% Nacl containing 300 n mol ml^{-1} oxygen, 20°C.

time. Apparently some processes terminate; Bailey and Shimokomaki (7) have shown that while aldehyde derived cross-links in young rat, bovine and human tissues change with age the process is complete by maturity. A further unanswered question is whether in vivo and in vitro aging are the same process.

In a recent paper (3) we presented evidence that human and rat tendon collagen age at much the same rate in vivo. Further, the rate of aging in vitro could be greatly increased, by about the same amount for both collagens, by increasing the concentration of free oxygen in the incubating solution compared with the amount available in the tissue fluid. Also, the rate of aging in vitro could be brought close to the in vivo rate by adjusting the free oxygen concentration to body tissue

level. These effects of oxygen are summarised in Fig.1 where isometric melting curves of rat tail tendon from an animal aged 40 days at death are shown after incubation at 20°C for 180 days in saline at two different concentrations of oxygen.[1] Curve A is the melting curve for the tendon with only in vivo aging, curve B that for the tendon incubated with 60 n mol ml^{-1} oxygen (tissue fluid level), while curve C was incubated with 300 n mol ml^{-1} of oxygen. Curve C is similar to the curve which would be obtained with tendon from a rat killed at 40 + 180 days (3).

We now report further evidence, based upon mechanical experiments using three different collagens, which supports the view that free oxygen plays a significant role in the aging process, both in vivo and vitro.

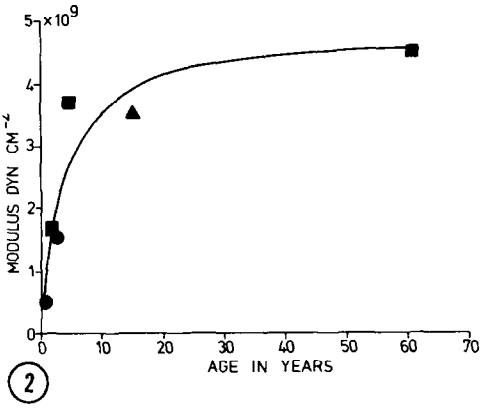
Methods

The extent of aging was gauged by simple load/extension curves determined on collagen fibres in physiological saline at 36°C. The fibres were extended in an Instron Extensometer at a rate of 1.2% min^{-1} until they broke.

The collagen fibres were taken from tendons of rat and dog tail and human wrist to yield mammalian collagens with similar amino-acid composition (and presumably similar cross-linking systems) but from animals with widely differing life-spans, namely 3, 15 and 80 years approximately. The samples were tested within a day or two after death so the results refer to in vivo aging effects. At least five fibres were tested from each sample and from the curves two measurements were made: the extension modulus of the linear part of the curve, and the maximum stress attained before breakage.

Results and Discussion Figs.2 and 3 give data derived from the load/extension curves plotted against animal age; in Fig.2 the modulus of extension, and in Fig.3 the maximum stress developed before the fibre

1. This data in non-graphical form, is referred to in Ref.3 and details of the experimental procedure are given there.



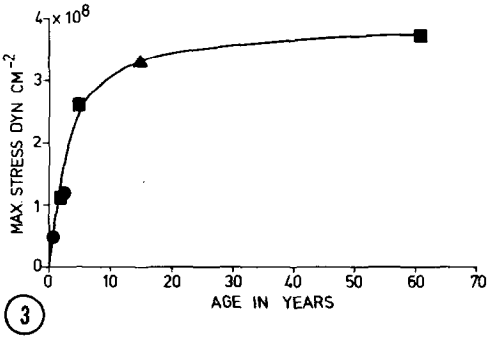


Fig.2. The modulus of the load/extension curve as a function of age for tendon from rat tail ●, human wrist ■ and dog tail ▲, extended in 0.9% Nacl at 36°C, at a rate of 1.2% min^{-1} until breakage occurred.

Fig.3. The stress at break attained in the load/extension curve as a function of age, for the same samples and conditions listed in the legend to Fig.2.

broke. It is apparent that both plots result in a curve which smoothly connects values from the collagen of the three species, and it seems that if the rat and dog lived longer the aging parameters of their collagen would follow the same curve as for human collagen. In other words the three collagens appear to age (as far as mechanical properties are concerned) in a manner which seems to have no connection with life-span. As a check of this interpretation we performed load/extension experiments upon tail tendon taken from two rats killed at the age 50 days and 23 months and whose tendon had been aged in vitro for 2.5 years as described in Ref.3. According to our earlier findings the increased free oxygen available to the tendon under the in vitro condition would increase the aging rate so that the process would be completed well within the 2.5 years. The results obtained were in very good agreement with limiting values shown in Figs.2 and 3 for the young rat: (5.6×10^9 dynes cm^{-2} for modulus and 3.6×10^8 dynes cm^{-2} for stress at break), while for the older rat the modulus agreement is

excellent (4.8×10^9), and the agreement for stress at break is reasonable (2.4×10^8).

Our earlier (3) finding that the isometric melting curve of 54 year old human wrist tendon was not altered by 7 months in vitro aging, whereas the melting curve for 5.5 year old human tendon showed a marked alteration toward the melting characteristics of "old" tendon after 4 months of in vitro aging, is further support of this hypothesis.

Recent work (8) has shown that the rate of insolubilisation of reprecipitated collagen fibres is also dependent upon oxygen. Furthermore, the authors showed that the changes with time could not be due to the conversion of reducible aldimine cross-links to more stable forms because the decrease in these bonds took place with or without oxygen.[2]

All the above results suggest that the age-related changes in the properties of collagenous tissues could be due to two distinct categories of cross-linking. One is the genetically determined system concerned with the formation, growth, maturation and repair of tissues. The other is a continuous oxygen-dependent mechanism resulting solely from the constant background of free oxygen in the tissue fluid. Whatever the nature of this mechanism, its end result is consistent with a cross-linking reaction.

Finally, there is a general problem associated with the aging of connective tissue for which the present work has relevance. This is the problem raised by the evidence that at least some of the genetically determined cross-linking reactions, if not all (6,7) are completed by maturity, yet the aging process continues throughout life. In any case, there is no obvious biological reason why the tissues should continue to be "toughened"—in fact most aging effects are considered to be undesirable.

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2. It should be pointed out here that insolubility, mechanical strength and the magnitude of the stress and temperature attained before breakdown of the fibre in an isometric melting experiment, are all indices of the co-valent cross-linking state of the fibre i.e. these parameters increase if the number and/or the thermal and mechanical stability of the cross-links increases.

The unavoidable oxygen-mediated reactions which we have postualted, would provide an explanation for this problem.

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