Application of Stress-Strain Behavior to Thermally Contracted Collagen from Epimysial Connective Tissues

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The molecular weight between covalent cross-links (Mc) in collagen from epimysial connective tissues was calculated, utilizing the kinetic theory of rubber elasticity. Epimysial connective tissues underwent osmotic swelling in neutral solution. In the course of the stress-strain measurements, two moieties were lost from the native collagen—i.e., water-soluble ground substance and heat labile collagen. The Mc values were affected by prior equilibration technique and by increased time in the 85° C. water bath. The Mc values for porcine epimysial connective tissue declined from 8.00×10^4 to 4.67×10^4, with the corresponding number of cross-links increasing from 4.09 to 7.73 per molecule as animal age increased from one week to five months. The Mc and cross-linking values for bovine epimysium were 1.42×10^4 and 21.31, respectively. Formaldehyde treatment reduced the Mc values to approximately 8.0×10^3.

Collagen is a fibrous protein which occurs as the principle constituent of connective tissues. The native protein is a tough, relatively inelastic material. Upon denaturation by heat in the presence of water, the fiber contracts to approximately one quarter of its initial length and imbibes large quantities of the liquid medium (Wiederhorn and Reardon, 1952). At the shrinkage temperature, there are no longer sufficient bonds to maintain the oriented structure, and a random amorphous network tends to form in which the surviving cross-links become the junction points (Cater, 1963). Once hydrothermal shrinkage has taken place, more water flows into the interstices between the disorganized protein chains and the swollen mass becomes rubberlike and highly elastic.

Flory and Rehner (1943) have developed an equation based on the statistical theory of rubberlike elasticity, which defines the force-extension relationship for a swollen elastomer. Wiederhorn and Reardon (1952) have shown that thermally contracted collagen obeys the kinetic theory of rubber elasticity. From the stress-strain behavior of a thermally contracted sample, a molecular weight between points of cross-linking can be determined. From this quantity it is possible to calculate the number of cross-links per unit volume of protein (Wiederhorn *et al.*, 1953).

The changes observed in collagen in relation to advancing biological age, species, and certain diseases are actually manifestations of the extent of cross-linking in the collagen molecule (Piez *et al.*, 1966; Verzar, 1964). However, very little work has been reported on the stress-strain approach of measuring the heat-stable bonds in native collagen. Therefore, the present study was initiated to determine the cross-link characteristics of native epimysial connective tissue collagen.

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MATERIALS AND METHODS

Sample Treatment. The epimysium from six 5-month-old market pigs, two 1-week-old pigs, three 7-week-old pigs, and two commercial grade cows were utilized in this study. The epimysium from the anterior portion of the *longissimus dorsi* muscle in the region of the 3rd and 4th rib at 48 hours post-mortem was removed and dissected free of adhering fat and muscle. The samples were frozen in liquid nitrogen and stored at −20° C. for subsequent physical studies.

Stress-Strain Apparatus. Prior to making stress-strain determinations, samples were thawed and then soaked for periods of 0.5 to 2 hours in either distilled water or 0.9% saline solution. Samples were placed between the two clamps of the stress-strain apparatus. The upper clamp was attached to a Statham, Model UL 5, microscale accessory and universal transducing cell. The universal cell was attached to a Statham precision readout (Model UR 4). The lower clamp was mounted on a rod which could be moved vertically, thus extending the sample. The distance between the upper and lower clamp could be recorded accurately by means of a dial micrometer.

The apparatus was arranged so that a constant temperature water bath could be raised to the height of the two clamps, thus positioning the sample so that it was immersed in 85° C. water.

Stress-Strain Measurements and Calculations. The samples were equilibrated at 85° C. for periods from 0.5 to 2.0 hours. The specimen was then extended by 15 to 25% of the unstressed length in six to eight equal steps. At each stage the corresponding equilibrium tension was recorded. Finally the sample was removed from the apparatus by slicing through close to the face of each clamp with a sharp knife. The samples were blotted lightly and the wet weight determined. Samples were then dried 24 hours at 90° C., and the dry weight was recorded. The molecular weight between cross-

links was calculated according to a modification of the equation of Wiederhorn *et al.* (1953):

$$Mc = \frac{V^{1/3}}{F} RTd \left(\alpha - \frac{1}{\alpha^2}\right)$$

Where:

F = retractive force per unit cross-section
α = relative elongation of the sample—i.e., the ratio of the stretched length to the initial length
d = density of the unswollen fiber (1.33)
T = temperature, °K.
R = gas constant
Mc = average molecular weight of the chains between cross-links
V = volume fraction of the rubber constituent of the sample

The cross-sectional area was calculated from the dry weight, wet weight, and length of the unstressed sample, assuming a density of 1.33 for the dry collagen and 1.00 for the water fraction. The volume fraction was calculated from the volume of the wet and the dry samples.

RESULTS AND DISCUSSION

Equilibration Treatment. During initial stress-strain determinations, swelling and dimensional changes were noted in the samples equilibrated in distilled water. Further studies (Table I) revealed that the samples placed in both acetic acid and distilled water developed the characteristic appearance attributed to osmotic swelling (Gustavson, 1956). The alterations in appearance and structure were materially less when the samples were placed in 0.9% saline.

Table I. Neutral Swelling Characteristics of Epimysial Connective Tissue[a]

Animal No.	Two-Hour Treatment	Water Uptake[b]	Length after Soaking[c]	Width after Soaking[c]
11	Distilled water	6.27	92.67	128.67
3	Distilled water	9.29	93.17	132.00
4	Distilled water	10.00	82.00	255.67
4	0.9% saline	0.06	97.00	110.67
4	3*M* acetic acid	16.00	86.33	372.00

[a] Connective tissue samples were from 5-month-old market weight pigs. [b] Grams of water per gram of tissue. [c] Per cent of initial length or width.

Table II. Effect of Various Soaking and Heating Treatments on the Dry Matter Content of Epimysial Connective Tissues[a]

Treatment	Dry Matter, %	Dry Matter Loss, %
Native epimysium	39.50	—
15 hr. in 1*M* NaCl	35.92	3.58
2 hr. in distilled water	35.78	3.72
15 hr. in 1*M* NaCl and 2 hr. in distilled water	34.91	4.09
1 hr. in 85° C. distilled water	20.26	19.24
0.5 hr. in 85° C. distilled water	20.14	19.36
2 hr. in distilled water, then 0.5 hr. in 85° C. distilled water	14.90	24.60
24 hr. in formaldehyde	33.00	6.50

[a] Connective tissue samples were from normal 5-month-old market weight pigs.

The samples placed in water showed a marked loss in dry matter during the soaking period (Table II). The loss in dry matter can probably be explained by the extraction of ground substance from the connective tissues, since soaking in 1*M* sodium chloride and in 0.9% saline resulted in similar losses in dry matter (Lowther *et al.*, 1967).

Heating in distilled water also resulted in large losses in dry matter. The amount lost was essentially the same, regardless of whether the sample was heated for 0.5 or 1 hour. Thus, in the course of stress-strain measurements, two moieties appeared to be lost from the native collagen—i.e., the water-soluble ground substance and the heat labile collagen (Verzar, 1964). The soaking treatment prior to heat shrinkage also affected the results of the stress-strain determinations (Table III). A significant increase in Mc values was noted with increased equilibration periods in distilled water. Conversely, stress-strain Mc values were relatively constant in samples soaked for extended periods in 0.9% saline.

The reason for the decrease in cross-linking by the neutral swollen epimysial collagen is not clear. However, similar phenomena have been observed by the other workers. For example, Harkness and Harkness (1965) reported a decrease in tensile strength in rat skin collagen incubated in weak buffer solution. These authors suggested that this might be the result of an internal pH change produced by cellular activity in a weakly buffered external medium. In addition, Verzar (1964) concluded that the decrease in thermal shrinkage temperature observed in rat tail tendon immersed in distilled water for 10 minutes was due to the withdrawal of electrolytes.

An increase in the molecular weight between cross-links also occurred with increasing holding times in 85° C. distilled water (Table IV). Although this first suggested that the samples continued to shrink as the soaking period was increased, this was obviously not the case, as can be seen on comparing contracted lengths after various periods of soaking (Table IV). The data in Table II also indicated that time in the 85° C. water bath did not affect the release of heat-soluble components and, therefore, should not affect the molecular weight calculations. The only dimensional parameter which varied with time was the length the sample attained at a given retractive force (Table IV). This implies that cross-links were broken with increased periods of time in the 85° C. bath. This conclusion is substantiated by Verzar (1964), who reported that strong covalent cross-links were destroyed if collagen samples are held at a level at or above the shrinkage temperature. The number of cross-links broken per unit time apparently reached a minimum after 1 hour at 85° C.

Stress-Strain Data. A graph of extension force (F) per unit cross-sectional area against $\alpha - \frac{1}{\alpha^2}$ is given in Figure 1. Provided hydrothermally denatured collagen follows the theory of ideal rubber elasticity, which defines the force-extension relationship for a swollen

Table III. Changes in Epimysial Connective Tissue Characteristics with Increased Equilibration Time[a]

Time, Hr.[b]	Mc × 10^4 [c]	Cross-links per Molecule[d]
0.5	1.20	24.99
1.0	1.31	22.88
1.5	1.49	20.04
2.0	1.76	16.99
2.5	2.25	13.36

[a] Connective tissue samples were from commercial grade beef.
[b] Samples were equilibrated in distilled water for period indicated before subjection to 0.5 hr. in 85° C. distilled water.
[c] Mc = molecular weight between cross-links.
[d] Assuming a molecular weight of 300,000 for the collagen molecule.

Table IV. Changes in Epimysial Connective Tissue Characteristics with Increased Time in 85° C. Distilled Water[a]

Hours in 85° C. Water Bath[b]	Contracted Length, Inch	Length to Attain 5.43 Grams of Force, Inch	Mc × 10^4 [c]	Cross-links per Molecule[d]	Number of Cross-links Broken
0.25	0.156	0.190	2.04	14.74	0.00
0.50	0.160	0.197	2.43	12.33	2.41
0.75	0.163	0.204	2.81	10.69	1.31
1.00	0.163	0.207	2.96	10.12	0.57
1.25	0.160	0.210	3.11	9.63	0.49
1.50	0.160	0.214	3.31	9.06	0.57
1.75	0.160	0.218	3.51	8.55	0.51
2.00	0.160	0.222	3.69	8.12	0.43

[a] Connective tissue samples were obtained from commercial grade beef.
[b] Samples subjected to equilibration in distilled water for 2.5 hr.
[c] Mc = molecular weight between cross-links.
[d] Assuming a molecular weight of 300,000 for the collagen molecule.

Table V. Effect of Biological Aging and Species on the Molecular Weight Between Cross-links in Epimysial Connective Tissues[a]

Source	Number of Animals	Mc × 10^4 [b]	Cross-links per Molecule[c]
1-week-old pig	2	8.00	4.09
7-week-old pig	3	6.58	4.81
5-month-old pig	6	4.67	7.73
Commercial grade beef	2	1.42	21.31

[a] Samples equilibrated for 1.0 hr. in 0.9% saline and then subjected to 85° C. distilled water for 1.0 hr.
[b] Mc = molecular weight between cross-links.
[c] Assuming a molecular weight of 300,000 for the collagen molecule.

elastomer, such a graph should be a straight line passing through the origin (Cater, 1963). Figure 1 shows that the response for epimysial connective tissues was linear up to an $\alpha - \frac{1}{\alpha^2}$ value of approximately 55 × 10^{-2}, indicating that the behavior of epimysial connective tissue over this range was identical to that of an ideal rubber.

The results of a study on the effects of biological aging on the covalent cross-linking in epimysial connective tissues are shown in Table V. These results clearly show a decrease in the molecular weight between cross-links and a corresponding increase in the number of cross-links as the animal becomes older.

The small molecular weight between cross-links and resultant large number of cross-links observed in the bovine epimysium probably illustrates a species difference. However, these values may also reflect differences in cross-linking due to the aging process.

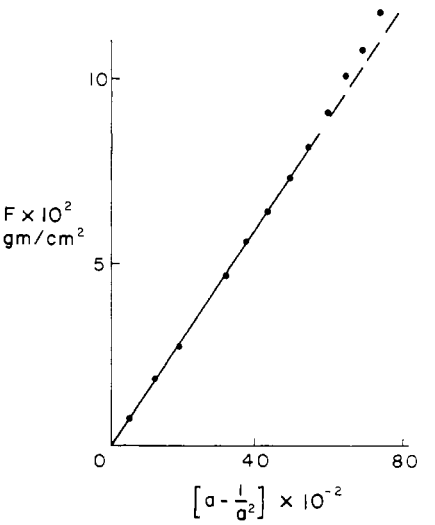

Figure 1. Plot of extension force (F) per unit of cross-sectional area against $\left[\alpha - \frac{1}{\alpha^2}\right]$

A comparison with values reported in the literature is difficult, due mainly to the limited amount of work reported and the variety of sources of collagen utilized. Wiederhorn and Reardon (1952) reported a Mc value of 5.5 × 10^4 for kangaroo tail tendon. Cater (1963), however, found a value of 19.9 × 10^4 for freshly isolated kangaroo tail tendon, as compared to a value of 5.5 × 10^4 after preparation for surgical sutures. Cater (1963) also reported a value of 6.4 × 10^4 for wallaby tail tendon that had been acetone-dehydrated and stored in the air-dry state for a number of years.

The only study pertaining to stress-strain studies in relation to animal age was reported by Kulonen *et al.* (1963). They found that the molecular weight between cross-links for rat skin was 15.0 × 10^4 at 3 months of age, 9.5 × 10^4 at 6 months, 4.5 × 10^4 at 12 months, and 3.7 × 10^4 at 24 months of age. These same workers found Mc values of 5.0 × 10^4 to 6.5 × 10^4 in rat tail tendon, regardless of age. When the Mc values found for the 7-week-old pigs were subjected to the calculations outlined by Wiederhorn and Reardon (1952), a value of 675 A. was obtained for a unit with a molecular weight of 6.58 × 10^4. The similarities between this value and the major collagen fiber repeat distance

Table VI. Effect of Formaldehyde Tanning on the Molecular Weight Between Cross-links in Epimysial Connective Tissue[a]

Incubation Time in 0.1% Formaldehyde, Hr.	Mc × 10^3 [b]	Cross-links per Molecule[c]
4	7.87	38.14
24	8.27	36.27
48	8.33	36.01

[a] Connective tissue samples were from normal 5-month-old market weight pigs.
[b] Mc = molecular weight between cross-links.
[c] Assuming a molecular weight of 300,000 for the collagen molecule.

(640 to 700 A.) indicate that the molecular weight unit determined by this procedure seems to have a basis in the structure of native collagen.

The changes in molecular weight between cross-links due to formaldehyde treatment of epimysial connective tissues are shown in Table VI. Formaldehyde treatment decreased the molecular weight between cross-links from 4.67×10^4 (Table V) to approximately 8.0×10^3. These values are lower than those reported by Wiederhorn and Reardon (1952), who found the Mc values to be 1.5×10^4 for formaldehyde-tanned kangaroo tail tendon. Cater (1963), however, found a value of 8.9×10^3 for kangaroo tail tendon, and a value of 2.68×10^4 for calf tendon. Apparently the number of cross-links introduced by formaldehyde are affected by age and species.

The results of this study have shown that epimysial collagen follows the theory of ideal rubber elasticity. Variation in Mc and cross-linking values were noted with increasing animal age, species, and formaldehyde treatment. Therefore, it is suggested that the stress-strain technique may provide a valuable tool for measuring variations in the cross-linking characteristics of native connective tissues.

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