Experimental Section

Gerontology *26:* 61–67 (1980)

Age-Related Changes in Rat Muscle Collagen

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Key Words. Rat · Collagen · Hydroxyproline · Cross-linking · Slow muscle · Fast muscle · Cardiac muscle

Abstract. Age-related studies of collagen in slow, fast and cardiac muscle of the rat indicate that different fractions of collagen as well as total collagen content vary with age and type of muscle. The total collagen level increases by 30% in slow, 40% in fast and 50% in cardiac muscle as age advances from 5 to 25 months. Collagen of the muscles of old animals is less susceptible to the collagen-degrading enzyme when compared to the young, and the activity of the enzyme decreases significantly with age. The decrease in (1) the solubility of collagen; (2) the amount of hydroxyproline released at 65 °C, and (3) the increase in the resistance of collagen to the degrading enzyme seen with aging, indicates that the stability of collagen increases in these muscles with aging.

Results of the study of age-related changes of different fractions of collagen of slow, fast and cardiac muscle of the rat are presented in this paper.

Materials and Methods

Soleus (slow, red), extensor digitorum longus (fast, white) and cardiac muscle of albino rats of the Wistar strain aged 5, 10, 15, 20 and 25 months were used for the present study. Salt-soluble (0.5 *M* NaCl, pH 7.4), acid-soluble (0.1 *M* acetic acid) and insoluble collagen fractions were extracted and purified according to *Jackson and Cleary* (1967). Hydroxyproline content was estimated according to *Neuman and Logan* (1950) as modified by *Leach* (1960) and the values were multiplied by 7.46, the conversion factor for collagen. Degree of thermic liberation of hydroxyproline was estimated according to the method of *Verzár* (1960).

To determine the collagen-degrading enzyme activity, the muscles were homogenized in ice-cold 0.02 *M* Tris buffer at pH 7.4 and centrifuged at 7,000 rpm for 20 min at 0 °C. 2 ml of the supernatant was incubated with the substrate (collagen) at room temperature for 3 h. At the end of the incubation period the activity was stopped by adding 1 ml of 10% TCA. Further, the samples were centrifuged at 4,000 rpm for 20 min, the supernatant was hydrolysed in 6 *N* HCl at 15 lb pressure for 2 h and hydroxyproline was estimated. Protein content of the supernatant was estimated

according to *Layne* (1957). An unincubated, TCA-denatured homogenate served as control.

The substrates used for the study were crude soluble collagen extracted from muscles of 3- and 20-month-old rats, and purified soluble and insoluble collagen extracted from bovine Achilles tendon (Sigma chemicals). The soluble collagen was extracted from the muscles according to *Rubins et al.* (1964).

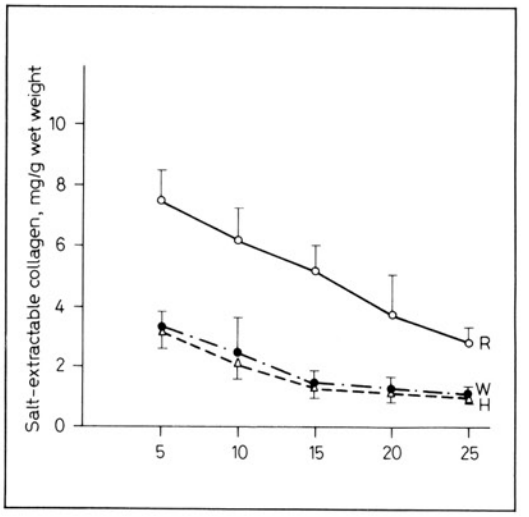
Results and Discussion

The extractability of different fractions of collagen varied with the type of muscle and age. The amount of salt-soluble collagen was more in red muscle compared to white and cardiac in all the five age groups studied, and there was no significant difference in the salt-soluble collagen content between white and cardiac (fig. 1). The salt-soluble collagen decreases with advance in age in all the three muscles (63% in red, 70% in white and cardiac). This may be either due to a decrease in the rate of synthesis, an increase in the rate of turnover, or variation in the conversion rate of salt-soluble to acid-soluble/insoluble collagen. Acid-soluble collagen increases up to 15 months of age in red and white muscle and up to 20 months in cardiac (fig. 2). This indicates that the rate of formation of acid-soluble collagen may be greater than its rate of conversion to insoluble collagen and also its breakdown. In red muscle there is a decrease in acid-soluble collagen after 15 months of age which may be due to the rate of conversion being more than the rate of synthesis, while in white and cardiac muscle the acid-soluble collagen content remains almost constant between 20 and 25 months of age. *Burtzow and Eichhorn* (1968) suggested that the acid-soluble collagen of the muscle of old animals may be a form which does not change into insoluble collagen and is formed mainly by intramolecular cross-linking.

Insoluble collagen shows a significant age-related increase, 147% in red, 103% in white and 186% in cardiac muscle, between 5 and 25 months (fig. 3). Though the total protein content decreases with increase in age (*Mohan and Radha,* 1975), the fibrous protein collagen shows an age-related increase in all the three muscles. The sarcoplasmic protein degradation rate increases by 50–70% in all the three muscles between 5 and 25 months of age (*Mohan and Radha,* 1978) while the total collagen level increases by 30% in red, 40% in white and 50% in cardiac muscle, which may explain the decline seen in total protein content (table I).

Schaub (1963) has shown an increase in total collagen content by 50% in abdominal muscle and 100% in hindleg and back muscles of the rat during aging. The total collagen content has also been shown to increase in denervated (*Vecchioni and Tartarini,* 1961), senile dystrophic (*Lowry et al.,* 1942; *Dreyfus et al.,* 1954), and work-induced hypertrophic (*Bartsova et al.,* 1969) muscles of rats. The present results which show an increase in total collagen in the cardiac muscle is similar to the results of *Schaub* (1964a) and *Knorring* (1970). However, other investigators (*Oken and Boucek,* 1957; *Laves and Correl,* 1960; *Montfort and Perez-Tamayo,* 1962; *Wegelius and Knorring,* 1964) have observed no definite postnatal change in the total collagen content of the myocardium.

The present data shows that with advance in age there is progressive insolubilization of collagen of all the three muscles, which is indicated by the ratio of soluble/insoluble collagen. The ratio is greater in the muscles of young animals compared to the old (table I). A similar trend has been reported with reference to the skin of man (*Kohn and Rollerson,* 1959; *Verzár,* 1964), rat (*Wirtschafter and Bentley,*



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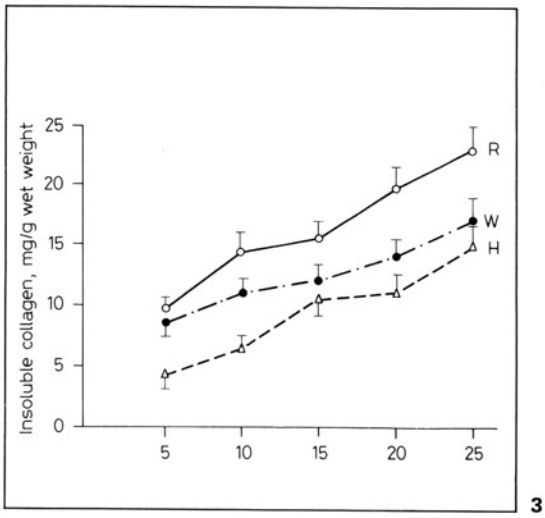

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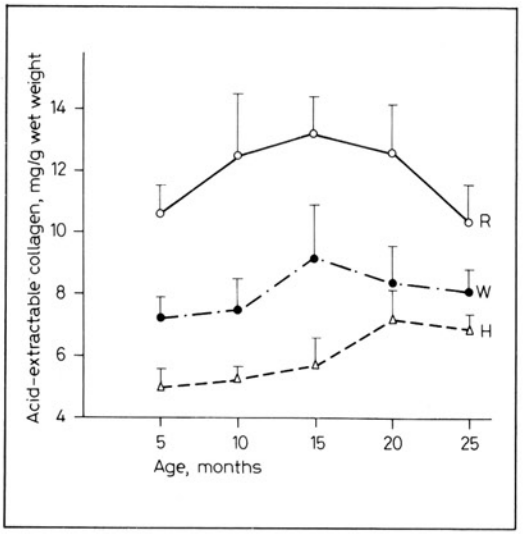

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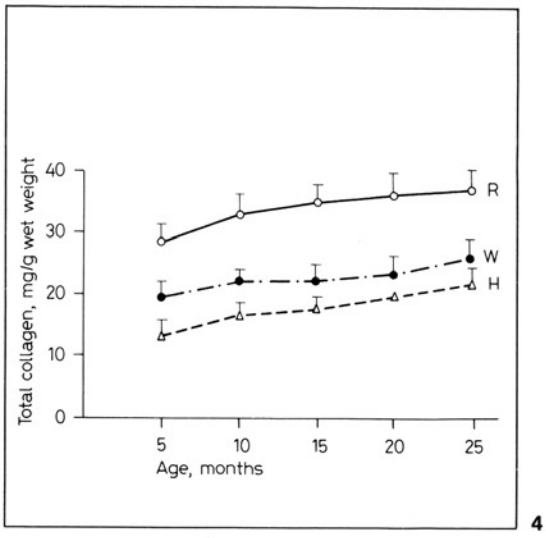

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Fig. 1–4. 0.5 *M* NaCl soluble (fig. 1), 0.1 *M* acetic acid soluble (fig. 2), insoluble (fig. 3) and total (fig. 4) collagen level. R = Red muscle; W = white muscle; H = cardiac muscle.

1962; *Heikkinen and Kulonen,* 1968; *Tsurufuji and Ohuchi,* 1968), rabbit (*Nageotte and Guyon,* 1934; *Nimni et al.,* 1965), tendon of rat (*Verzár,* 1960; *Everitt et al.,* 1970) and lizard (*Panigraphy and Patnaik,* 1976).

Figure 5 shows the age-related decrease in the amount of free hydroxyproline released at 65 °C (55% in red and 48% in white and cardiac muscle between 5 and 25 months) indicating the decrease in labile collagen which can be either due to conversion of weaker bonds to stable cross-links or increase in the number of stable cross-links. The amount of free hydroxyproline released at 65 °C from the muscles of

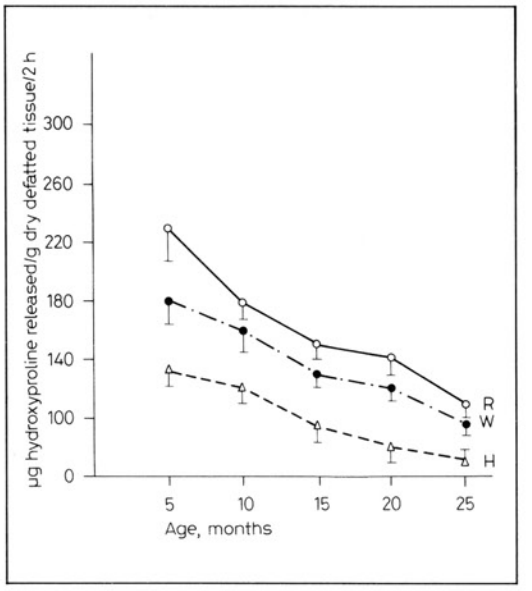
Table I

Nature of muscle		Age, months				
		5	10	15	20	25
Red	total protein	154.5 ± 7.5	159.6 ± 11.1	146.9 ± 11.5	140.0 ± 12.8	135.3 ± 6.4
	soluble collagen	18.18 ± 1.74	18.67 ± 3.68	18.47 ± 1.91	16.34 ± 3.1	13.12 ± 1.67
	insoluble collagen	9.57 ± 1.62	14.18 ± 2.5	16.16 ± 1.69	19.42 ± 1.72	23.7 ± 2.1
	soluble/ insoluble	1.899	1.316	1.132	0.841	0.553
White	total protein	139.7 ± 8.4	134.7 ± 10.8	120.3 ± 10.3	109.9 ± 6.3	106.3 ± 6.0
	soluble collagen	10.65 ± 0.94	9.85 ± 2.3	10.59 ± 1.98	9.53 ± 1.62	9.06 ± 1.07
	insoluble collagen	8.48 ± 0.96	11.0 ± 1.23	11.53 ± 1.34	13.54 ± 1.25	17.12 ± 2.42
	soluble/ insoluble	1.255	0.995	0.918	0.703	0.529
Cardiac	total protein	145.7 ± 9.2	153.2 ± 12.8	142.3 ± 9.2	134.8 ± 7.7	129.0 ± 6.7
	soluble collagen	8.18 ± 0.95	7.21 ± 0.80	7.01 ± 1.04	8.40 ± 1.41	7.62 ± 0.65
	insoluble collagen	4.27 ± 0.62	6.44 ± 0.66	10.57 ± 0.91	11.07 ± 1.21	15.15 ± 2.0
	soluble/ insoluble	1.915	1.119	0.663	0.658	0.502

Total protein, soluble and insoluble collagen values are represented as mg/g wet weight and are mean ± SD of 5 observations.

old animals is much less compared to that of young and also the time taken for the release of hydroxyproline is much more from the muscles of old animals compared to young which is suggestive of the stability of collagen with advance in age (fig. 6).

Activity of collagen-degrading enzyme decreases with age in all the three muscles with all the four substrates studied (fig. 7–10). When the substrate is crude soluble collagen from muscles of young animals, the enzyme activity is 5- to 6-fold greater than with that of collagen from old animals (fig. 7, 8). So also with purified soluble collagen from bovine achilles tendon the enzyme activity is greater (5-fold) than with purified insoluble collagen (fig. 9,



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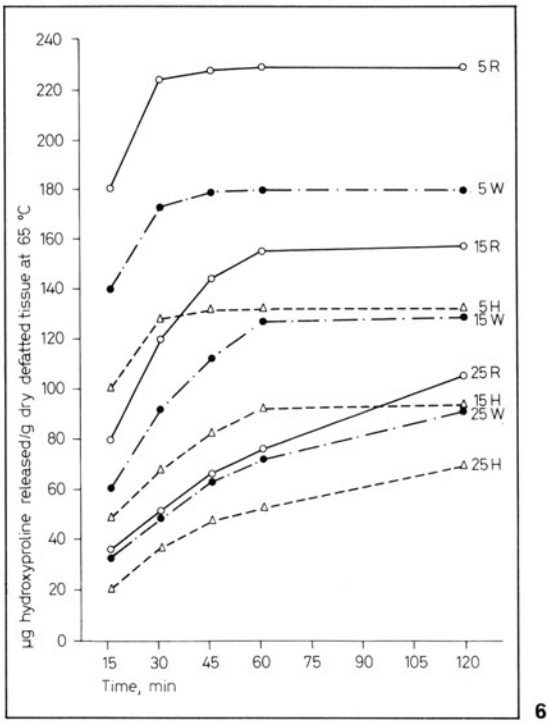

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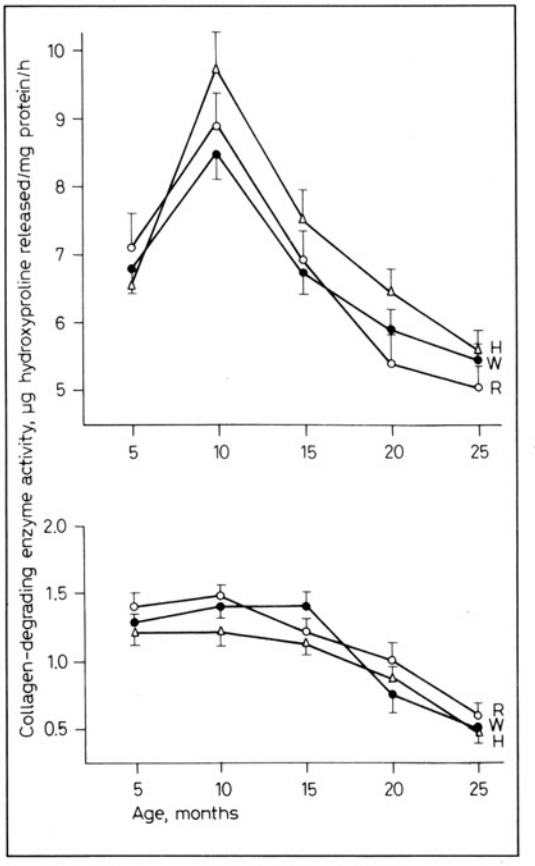
Fig. 5, 6. Amount of free hydroxyproline relased at 65 °C from red, white and cardiac muscle (fig. 5). Time course of release of hydroxyproline at 65 °C (fig. 6).

10). The collagen from muscles of old animals is less susceptible to the collagen-degrading enzyme compared to that of the young. The marked reduction in the activity of collagen-degrading enzyme in degrading soluble collagen or insoluble collagen reflects changes that occur with age in the physical properties of collagen due to cross-linking. A similar trend has been reported in the case of tendon of rat (*Schaub,* 1964b) and Calotes (*Panigraphy and Patnaik,* 1976). The present results strengthen the suggestion of *Hamlin and Kohn* (1972) that an increase in the resistance of collagen to collagenase digestion with advance in age could be a parameter to determine the biological age of an animal.

The present study has clearly shown that red, white and cardiac muscles differ in the level of total collagen as well as in acid-soluble, insoluble and labile collagen fractions. Since the fraction of collagen extracted with 0.5 *M* NaCl is considered to be freshly or newly synthesized, when its level is high in red muscle, it suggests that the rate of synthesis of new collagen is more in this muscle compared to white and cardiac. The results of collagen-degrading enzyme activity indicate that the rate of collagen degradation is almost the same in all the three muscles. Hence, the higher rate of collagen synthesis would account for the greater amount of total collagen seen in red muscle and this can be attributed to its function (maintaining the tonus, posture holding). Though the present results have shown that the changes in collagen as a function of age has a similar pattern in all the three muscles, the magnitude of change varies with the type of muscle.



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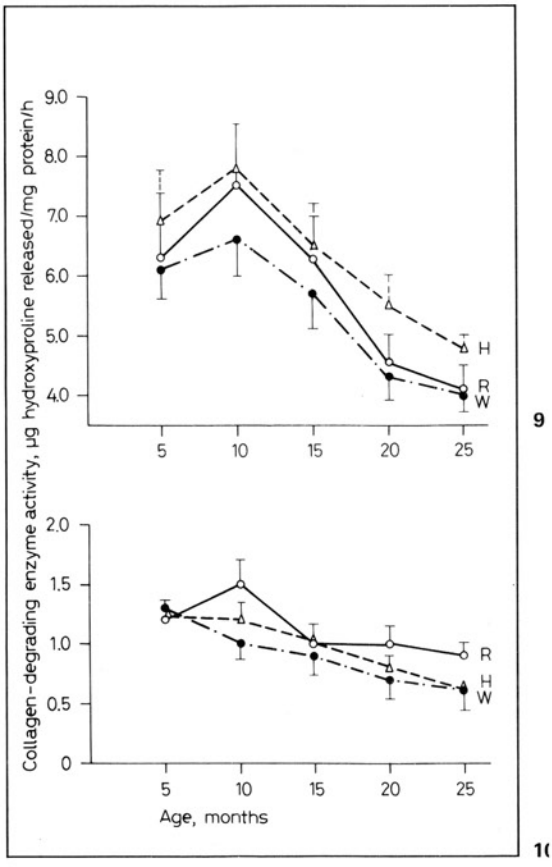

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Fig. 7–10. Collagen-degrading enzyme activity with crude soluble collagen extracted from muscles of 3-month-old (fig. 7) and 20-month-old (fig. 8) animals, and purified soluble (fig. 9) and insoluble (fig. 10) collagen from bovine Achilles tendon as substates. Values are represented as mean ± SD of 5 observations.

The present data on different fractions of collagen, decrease in the solubility, ratio of soluble/insoluble collagen, amount of hydroxyproline released at 65 °C and increased resistance of collagen to collagen-degrading enzyme activity with reference to slow, fast and cardiac muscle, strongly support the cross-linking theory which emphasizes a greater number of intra- and intermolecular cross-links with aging.

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Received: July 14, 1978
Accepted: April 18, 1979

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