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Age-related thermal stability and susceptibility to proteolysis of rat bone collagen

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The shrinkage temperature (T_s) and the pepsin-solubilizability of collagen fibrils in bone matrix obtained from decalcified femur diaphysis from 2-, 5-, 15- and 25-month-old rats were found to decrease with age. Digestion with human fibroblast collagenase dissolved less than half of the collagen, whereas sequential treatment by pepsin followed by collagenase resulted in its complete dissolution. This result shows that collagenase and a telopeptide-cleaving enzyme, when acting in an appropriate sequence, have a great potential for the degradation of bone collagen. The 'melting' profile of the pepsin-solubilized collagen showed a biphasic transition with transition peaks at 35.9 °C and 40.8 °C. With increasing age an increasing proportion of the collagen 'melted' in the transition peak at 35.9 °C (pre-transition), and the 'melting' temperature (T_m) of the collagen decreased in parallel with T_s in relation to age. Both T_s and T_m decreased by 3 °C in the age span investigated. The age-related change in T_s could therefore be accounted for by the decrease in molecular stability. The collagenase-cleavage products of the bone collagen obtained by the sequential treatment with pepsin and collagenase showed only one peak transition (at 35.1 °C), and the T_m for the products was independent of age. The results indicate that the pre-transition for the pepsin-solubilized collagen is due to an age-related destabilizing conformational change of the collagen molecule in the proximity of the collagenase-cleavage site. The age-related decrease in thermal stability may have implications for the mechanical strength and turnover of the bone collagen. In contrast with bone collagen, soft-tissue collagen showed neither the age-dependency of thermal stability nor the characteristic biphasic 'melting' profile.

INTRODUCTION

Collagen is the major component of the organic bone matrix, and the collagen present is type I with trace amounts of type V (Rhodes & Miller, 1978; Petrovic & Miller, 1984; Broek *et al.*, 1985). Bone type I collagen is identical in amino acid composition and sequence with type I collagen in soft tissues (Stoltz *et al.*, 1973). However, there are minor differences between soft-tissue collagen and bone collagen, resulting from post-translational chemical modifications of lysine residues (Fowler & Bailey, 1972; Stoltz *et al.*, 1973; Eyre & Glimcher, 1974; Eyre & Oguchi, 1980), as well as striking differences in physical and biochemical properties. In contrast with soft-tissue collagen, bone collagen does not swell in dilute acids (Bonar & Glimcher, 1981), and is only sparingly soluble in the native state (Glimcher & Krane, 1968).

In a previous paper we demonstrated another unique feature of bone collagen (Danielsen *et al.*, 1986): the mechanical strength of cortical-bone collagen decreased in relation to age and the strength of the collagen was not found to change upon incubation *in vitro*. In contrast, soft-tissue collagen increases in mechanical strength both during ageing and upon incubation *in vitro* (Danielsen *et al.*, 1986; Danielsen, 1987*a*).

In order to elucidate further the features of bone collagen that might be of importance to the mechanical quality and turnover of bone, in the present study rat bone collagen was analysed for sensitivity to enzymic digestion and thermal-denaturation characteristics in relation to age.

EXPERIMENTAL

Reagents

Pepsin (crystallized and freeze-dried) was purchased from Sigma Chemical Co. L-Cystine (Merck) was reagent grade. Other reagents used were analytical grade.

Experimental tissues

The femora were obtained from 2-, 5-, 15- and 25-month-old male Wistar rats. The bones were cleaned, and the diaphysial part of the bones was decalcified in 50 mM-disodium EDTA, pH 7.4, containing 1 mM-*p*-hydroxymercuribenzoate and 10 μM-phenylmethanesulphonyl fluoride for 3 weeks at 4 °C as previously described (Danielsen *et al.*, 1986). The decalcified bones were stored in liquid N_2 until required for analyses.

In order to explore for possible enzymic activity during the decalcification procedure, bone samples from 2-month-old rats were subjected to a different decalcification procedure. Frozen samples of bone were pulverized and then decalcified in 0.5 M-disodium EDTA, pH 7.4, for 16 h, either without addition of enzyme inhibitors, or with 1 mM-*p*-hydroxymercuribenzoate and 10 μM-phenylmethanesulphonyl fluoride added, or with the two above-mentioned inhibitors and 1 mM-1,10-phenanthroline added. After decalcification the bone samples were washed with cold distilled water and subjected to digestion with pepsin (described below).

Dorsal skin and tails obtained from male Wistar rats of different ages were stored at −20 °C.

Determination of thermal stability of the fibrils

Circular specimens, 2 mm in diameter punched out from 40 μm-thick and approx. 100 μm-thick freeze-sections of decalcified bone and skin respectively, were studied in five or six rats of each age group. The thermal stability of collagen fibrils was determined as the area shrinkage without tension during gradual heating (AS_T), and the shrinkage temperature (T_s) was calculated as the temperature for 50% of this area according to previously described principles (Danielsen, 1981). However, monitoring the area shrinkage by microphotographs at certain temperature intervals was replaced by another technique, using a Beckman DU spectrophotometer. The light-beam (λ = 240 nm) in the spectrophotometer was alternately passed through two circular apertures (2.4 mm in diameter) built into a water-cooled cell (Fig. 1). One aperture served as reference. In the other aperture the circular specimen was mounted soaking in 50 mM-Tris/HCl buffer, pH 7.4, deaerated in vacuum. The specimens

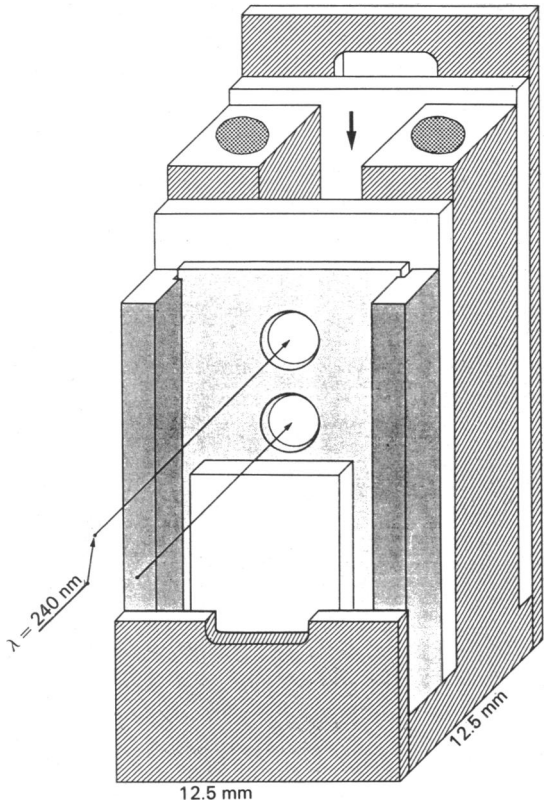

Fig. 1. Dissected view of the cell used for determination of shrinkage characteristics of bone and skin collagen fibrils

The front and back frames and the central block with canals for circulating thermostatically controlled water are made of brass. Three plates of quartz (Suprasil; Hellma) encase the central compartment ( ) containing deaerated distilled water in which the thermistor (Thermilinear component type 44201; Yellow Spring Instrument Co.) is situated and a specimen/buffer compartment comprising the two apertures through which the light is directed. The inside surfaces of the latter compartment are formed of the quartz plates and plastic (polyethylene, polystyrene). The distance between the quartz plates (and the thickness of the plastic plate with the apertures) was balanced to accommodate the increasing thickness and still prevent the crumbling of the specimen during shrinkage. The plastic plate with the apertures can be dismantled for cleaning.

completely absorbed the u.v. light, and the light passing through the aperture was only passing through the brim around the specimen. During gradual heating (2 °C/min) the specimen area was recorded by measuring the change in transmission through this brim. The alternate measurements in sample and reference apertures were controlled by an HP85 microcomputer, which also recorded corresponding temperatures and transmission values. The procedure described monitored the thermal shrinkage automatically, and the shrinkage curve and parameters could be calculated immediately after the shrinkage.

Proteinase digestion

Bone matrix, skin and tail tendon samples of different ages were represented by material from five or six rats of each age studied.

Pepsin digestion. Decalcified bone matrix was minced with a scalpel, suspended in cold distilled water and homogenized. The homogenization was performed in an Ultra-Turrax homogenizer at 50000 rev./min with the samples cooled in an ice bath. The pellet resulting from a subsequent centrifugation was freeze-dried. Weighed samples of the freeze-dried bone matrix were suspended in 0.5 M-acetic acid, and pepsin was added to give a pepsin/substrate weight ratio of 1:10. The suspension was incubated at 20 °C with magnet-stirring for 24 h. After incubation the pepsin/substrate mixture was dialysed against 50 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.4, and then either subjected to collagenase digestion (described below) or centrifuged (50000 *g* for 30 min at 2 °C).

Skin, cleaned for subcutaneous tissue, and tail tendons were minced with a scalpel and washed in cold 50 mM-Tris/HCl buffer, pH 7.4. The tissues were then homogenized in 0.5 M-acetic acid, and pepsin was added to give a pepsin/tissue wet-weight ratio of 1:10. The tissue samples were then incubated, dialysed and centrifuged as described above for bone matrix.

The pepsin-solubilizability of a sample was defined by the amount of hydroxyproline in the supernatant (resulting from the centrifugation following the incubation and dialysis) divided by the total amount of hydroxyproline in the supernatant and the precipitate.

The pepsin-solubilized collagen in the supernatant intended for electrophoresis and for determination of the thermal stability of the molecules was dialysed against 5 mM-acetic acid. Before this dialysis the skin collagen and tail tendon collagen were precipitated by addition of solid NaCl to 4 M, and the collected precipitates were then dissolved and dialysed against the acetic acid solution.

Collagenase digestion. The collagenase in serum-containing human fibroblast culture medium was isolated according to the procedures described by Stricklin *et al.* (1977). The collagenase-cleavage products resulting from the activity of this collagenase preparation have been characterized in a previous paper (Danielsen, 1987*b*).

Bone-matrix collagen was subjected to human fibroblast collagenase treatment as (1) the only enzyme treatment as well as (2) preceding and (3) following treatment with pepsin. The collagenase was activated by L-cystine as previously described (Danielsen, 1987*b*) and added to bone collagen samples suspended in 50 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.4, or 150 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.4. The suspensions were saturated with L-cystine and a drop of toluene was added. The enzyme/substrate mixtures were then incubated at 26 °C for 1 week, after which the incubation mixtures were chilled, acidified by addition of cold 0.5 M-acetic acid and dialysed against 50 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.4. After a subsequent centrifugation (50000 *g* for 30 min at 2 °C) the percentage of collagen solubilized was determined as described above under 'Pepsin digestion'.

Absorbance–temperature transitions of collagen molecules

Determination of the thermal stability by u.v. difference spectroscopy and calculation of smoothed 'melting' profiles were performed by the procedures previously described (Danielsen, 1982*a*, 1984). The thermal stability of collagen components was determined in 5 mM-acetic acid solutions containing 0.1–0.2 mg of collagen/ml.

Gel electrophoresis

SDS/PAGE was carried out at room temperature according to a previously described procedure (Danielsen, 1982*b*), based on that of Furthmayr & Timpl (1971), in 5% (w/v) acrylamide at 6 mA/tube until the tracking dye (Bromophenol Blue) was 1 cm from the gel bottom.

RESULTS

The T_s of the collagen fibrils in the bone matrix was found to

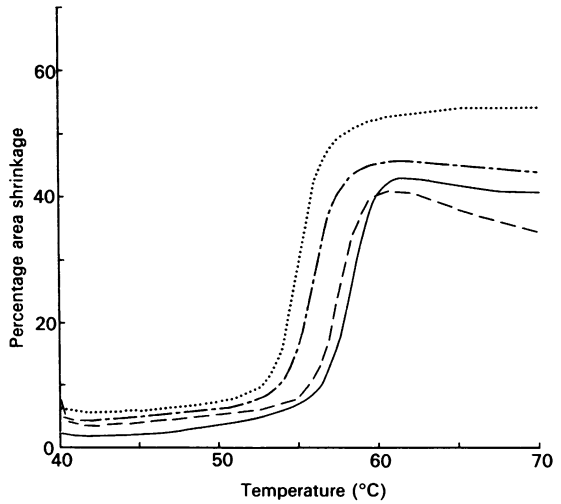

Fig. 2. Area shrinkage–temperature curves for bone collagen fibrils

The percentage area shrinkage is relative to the specimen area at 25 °C. The curve for each age group that is shown is the mean curve for six rats. The curve for each rat is the average curve of two specimens tested. ——, 2-month-old rats; – – –, 5-month-old rats; -·-·-, 15-month-old rats; ·····, 25-month-old rats.

Table 1. Thermal shrinkage characteristics of bone and skin collagen fibrils

AS_T is the percentage area shrinkage without tension during heating. Results are given as means ± S.D. ($n = 6$). Comparison among means was by one-way analysis of variance: $^*P < 0.01$.

Age (months)	Bone collagen fibrils		Skin collagen fibrils	
	T_s* (°C)	AS_T (%)	T_s* (°C)	AS_T (%)
2	58.2±0.4	38.3±7.2	63.4±1.0	22.7±11.0
5	57.6±0.5	37.7±5.9	65.1±0.7	17.4±6.4
15	56.1±0.4	39.7±4.6	64.8±0.4	15.5±5.1
25	55.2±0.4	44.6±8.5	64.3±0.4	25.0±8.0

decrease with age (Fig. 2 and Table 1), whereas the skin collagen fibrils obtained from the same rats showed minor, non-age-related, variation in T_s. The percentage area shrinkage was not found to change with age either for bone collagen or for skin collagen.

In order to characterize the bone collagen further, different methods for dissolution of the collagen were tried (Table 2). The pepsin-solubilizability was found to decrease from about 80% to 50% from 2 months to 25 months of age. Human fibroblast collagenase solubilized less than half of the bone collagen, whereas sequential treatment of the collagen by pepsin followed by collagenase dissolved all of it. The reverse sequence of pepsin and collagenase treatment was found to result in a dissolution of collagen corresponding to that obtained by pepsin treatment alone.

The 'melting' profiles of the pepsin-solubilized bone collagen showed a characteristic biphasic transition with peak transition at 35.9 °C and 40.8 °C (Fig. 3*a*). The height of the pre-transition (at 35.9 °C) was found to increase with age. This changing height of the pre-transition resulted in an age-related decrease in the calculated 'melting' temperature (T_m) (Table 3). The age-related decrease of T_m was found to be linearly related to the decrease in T_s.

In comparison with the results obtained after the decalcification procedure applied on the bones from rats of different ages, the decalcification of bone from 2-month-old rats that in addition was performed within a significantly shortened (30-fold) time period was not found to influence the 'melting' profile or the T_m of the bone collagen, irrespective of the presence of different enzyme inhibitors.

The 'melting' profiles of skin collagen and tail tendon collagen from 2-month-old, 3-month-old, 15-month-old and 25-month-old rats showed only a slight elevation at the temperature interval corresponding to the pre-transition for the bone collagen. The T_m values of skin collagen and tail tendon collagen, which represented the total amounts of tissue collagen obtained by complete dissolution by means of the pepsin treatment performed, were independent of age and determined to 39.1–39.3 °C and 39.3–39.5 °C respectively.

The thermal stability (T_m) of bone collagen completely solubilized by sequential treatment with pepsin and human fibroblast collagenase was independent of age (Table 3). The 'melting' profiles of the collagenase-cleavage products showed no pre-transition and did not show age-related variability (Fig. 3*b*), and therefore no attempt was made to separate the cleavage products as previously done (Danielsen, 1987*b*).

Scans of SDS/PAGE of pepsin-solubilized and pepsin/collagenase-solubilized bone collagen are shown in Fig. 4. About 12% of the components with mobility corresponding to that of α-chains resisted the collagenase digestion. These components might be type V collagen chains, which are known to be resistant to vertebral collagenase (Mainardi *et al.*, 1980). An additional component (α_x) moving in front of the α_2(I)-chain was apparent for the pepsin-solubilized collagen. Following the collagenase

Table 2. Solubilization of rat bone collagen

The bone collagen was treated with pepsin in 0.5 M-acetic acid at 20 °C for 24 h and human fibroblast collagenase in 50 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.4 (or *150 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.4), at 26 °C for 1 week. Results are given as individual values or as means ± S.D., as appropriate.

Age (months)	Treatment...	Solubilization (%)				
		0.5 M-Acetic acid (20 °C, 24 h) ($n = 1$)	Pepsin ($n = 3$)	Pepsin followed by collagenase ($n = 2$)	Collagenase followed by pepsin ($n = 1$)	Collagenase ($n = 2$)
2		9	78±10	91, 99	85*	28,* 44
5		9	72±7	93, 99	75*	16,* 32
15		7	52±7	86, 99	53*	17,* 31
25		7	46±4	94, 99	51*	16,* 51

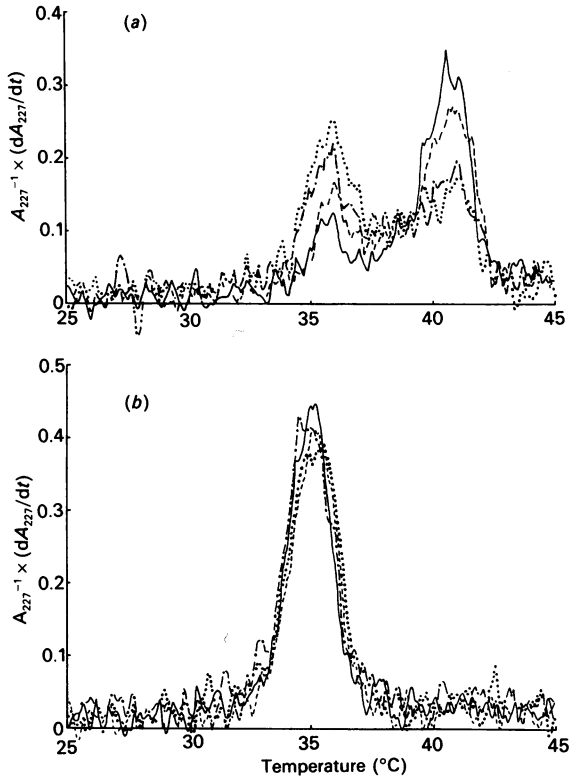

Fig. 3. 'Melting' profiles of bone collagen and collagenase-cleavage products thereof

Absorbance–temperature transitions were determined for bone collagen samples from rats of different ages that had been solubilized by (*a*) pepsin treatment and (*b*) sequential treatment with pepsin and collagenase. ——, 2-month-old rats; – – –, 5-month-old rats; ·—·, 15-month-old rats; ·····, 25-month-old rats.

Table 3. 'Melting' temperature and biochemical characterization of bone collagen and degradation fragments thereof

Percentage amounts (means ± S.D., $n = 4$) of components α_x and α_x^A were calculated from the colour-scan area (Fig. 4) as $(\alpha_x/85 \times 10^4)/[(100/85) \times \alpha_x + \alpha_1 + \alpha_2]$ and $(\alpha_x^A/60 \times 10^4)/[(100/60) \times \alpha_x^A + (100/75) \times (\alpha_1^A + \alpha_2^A)]$ respectively, assuming the following molecular masses: α_1- and α_2-chains, 100 kDa; components α_1^A and α_2^A, 75 kDa; component α_x, 85 kDa; component α_x^A, 60 kDa. The molecular masses of components α_x and α_x^A were calculated by subtraction of the average molecular-mass loss (15 kDa) relative to α_1- and α_2-chain components (see the Results section).

	Pepsin-solubilized collagen		Pepsin/collagenase-solubilized collagen	
Age (months)	T_m (°C)	α_x (%)	T_m (°C)	α_x^A (%)
2	40.0	6.4 ± 0.7	35.0	5.6 ± 0.9
5	39.8	8.6 ± 1.6	35.0	7.4 ± 2.5
15	37.8	16.0 ± 1.7	34.9	11.9 ± 1.9
25	36.9	16.3 ± 0.3	35.0	10.8 ± 0.8

treatment, a component (α_x^A) still moved in front of the α_2^A(I)-chain. By using the β-, α- and α^A-chains as molecular-mass standards, the α_x and α_x^A components had mobilities that corresponded to a molecular mass that was 11 kDa less than that of the α_2(I)-chain components (α_2 and α_2^A respectively) and that was 20 kDa less than that of the α_1(I)-chain components (α_1 and α_1^A respectively). The percentage colour-scan area of the additional components was higher for the collagen from the older rats (Table 3).

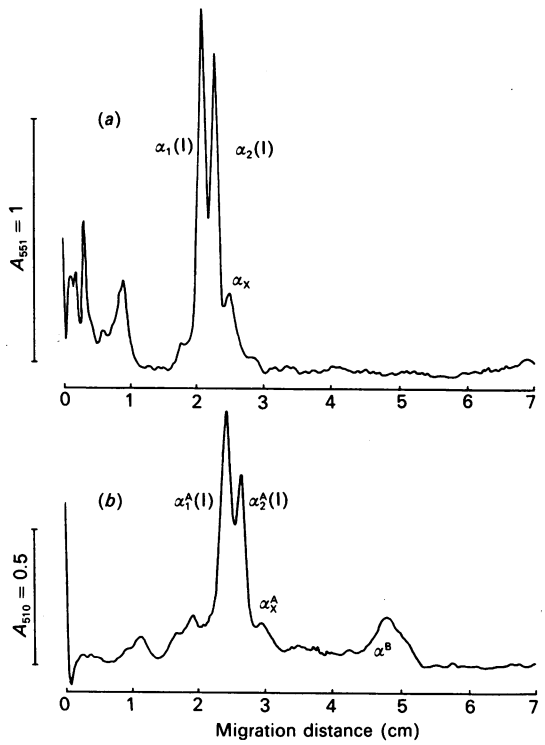

Fig. 4. Gel electrophoresis of bone collagen components from 25-month-old rats

The Figure shows scans of Coomassie Blue-stained gels with (*a*) pepsin-solubilized bone collagen and (*b*) pepsin/collagenase-solubilized bone collagen subjected to SDS/PAGE.

DISCUSSION

For bone collagen, the positive correlation between T_s and T_m indicates that the diminished fibrillar thermal stability is accounted for by a decrease in molecular stability. The age-related decrease in T_s is not related to the covalent cross-linking of the collagen, since this cross-linking becomes more extensive (the pepsin-solubilizability decreases) with age.

For bone collagen there is an age-related decrease in the mechanical strength (Danielsen *et al.*, 1986), and simultaneously there is a decrease in thermal stability, as found in the present study. Thus the decrease in thermal stability might be responsible for the decrease in mechanical strength with age. In contrast with bone collagen, there was no age-related decrease in the thermal stability of skin collagen and tail tendon collagen, and the mechanical strength of these soft-tissue collagens is known to increase with age (Vogel, 1978; Danielsen *et al.*, 1986; Danielsen & Andreassen, 1988).

The collagenase preparation applied in this study cleaved native type I and type III collagens (Danielsen, 1987*b*). The preparation had also some gelatinase activity (against gelatin of type I collagen), whereas cleavage of type V collagen and albumin was not discernible upon electrophoresis (C. C. Danielsen, unpublished work). Degradation of native TC^A (and possible TC^B) products, as reported by Overall & Sodek (1987), could conceivably affect the proteolysis of bone collagen and modify the properties of the collagenase-cleavage products, but has not been observed for the collagenase preparation applied.

There is evidence that minute amounts of collagenase are

present in mineralized bone and dentine (Sakamoto *et al.*, 1973; Dumas *et al.*, 1985; Eeckhout *et al.*, 1986). It is suggested that this collagenase is activated during bone lysis and participates in the bone resorption by degrading bone collagen after a preceding demineralization by osteoclasts (Eeckhout & Delaissé, 1988). The present results show that the concerted action of a telopeptide-cleaving enzyme (pepsin) and collagenase has a great potential for solubilizing the bone collagen. This finding indicates that collagenase, in combination with telopeptide-cleaving enzymes, possibly secreted by the osteoclasts, could play a significant role in the bone resorption process.

The collagenase-cleavage products (TC^A and TC^B) of the bone tropocollagen (TC) showed only one approximately symmetrical thermal transition with a peak transition at 35.1 °C. This is 5.7 °C below that of the main transition of the tropocollagen (obvious for the tropocollagen of 2-month-old and 5-month-old rats) (Fig. 3). These results are similar to those obtained for type I collagen from rat skin (Danielsen, 1987*b*).

Surprisingly, the pronounced pre-transition for the bone tropocollagen was not reflected in any way in the transition of the TC^A and TC^B products, and consequently this transition was unaffected by age. Samples of tropocollagen isolated from collagen fibrils incubated *in vitro* and from the acid-insoluble fraction of rat skin collagen showed a pre-transition similar to that observed for bone collagen (Danielsen, 1984). Similarly, for these tropocollagen samples neither the TC^A nor the TC^B product separated by the gel-filtration procedure previously described (Danielsen, 1987*b*) reflected the pre-transition of the parent tropocollagen (C. C. Danielsen, unpublished work).

Independently of the 'melting' pattern of the parent tropocollagen, the single transition peak for the collagenase-cleavage products indicates that the pre-transition of the tropocollagen is due to a destabilizing change in the proximity of the collagenase-cleavage site in the tropocollagen molecule. The peak temperature of the pre-transition of the tropocollagen was only 0.8 °C above that of the transition of the cleavage products. This finding agrees with the indication that the pre-transition is caused by a destabilizing change near the cleavage site in the tropocollagen molecule, provided that the helical portions of the tropocollagen molecule, extending in opposite direction from the cleavage site, 'melt' independently.

It is possible that the characteristic thermal stability of the bone collagen is caused by artifactual changes of the collagen, as pointed out by Kirsch *et al.* (1981). However, the following observations indicate that neither the preparation procedures nor the pepsin treatment applied in the present study are likely to result in the characteristic thermal denaturation of the bone collagen. Firstly, non-decalcified bone could be incubated at 37 °C for a period of 10 days, far exceeding the brief period taken to prepare the bone specimens for the present experiments, with only a slight decrease in T_s (by 0.8 °C) and no change in T_m (C. C. Danielsen, unpublished work). Secondly, this study indicates that the decalcification procedure applied is not critical for the results. Thirdly, T_s of the bone collagen fibrils was found to decrease with age without involvement of pepsin, and skin collagen and tail tendon collagen were treated with pepsin without showing the pronounced pre-transition or the age-related variability of 'melting' characteristics that bone collagen did.

However, the electrophoresis performed in the present study showed that collagen fragments existed in the bone collagen preparations obtained by the pepsin digestion (Fig. 4). For the tropocollagen one additional component (α_x-chain) was apparent, and following collagenase treatment this component seemed to appear as the component marked α_x^A in Fig. 4. Considering the electrophoretic mobility change following this treatment, the α_x-component is likely to be an α-chain that has lost an 11 kDa fragment or a 20 kDa fragment from its *N*-terminal portion, since the molecular mass of the α_x^A component corresponded to that of the α_x-chain minus that of the TC^B product. However, further biochemical characterization of the α_x and α_x^A components is necessary to confirm this relationship. The presence of the proteinase-cleavage fragments observed for the pepsin-solubilized collagen hardly explains the characteristic 'melting' pattern of the bone collagen, since the fraction of the α_x component cannot account for the fraction of collagen that 'melted' in the pre-transition. Other fragments, not evident in the electrophoresis performed, might be present. But the 'melting' profiles for the collagenase-cleavage products were found to be independent of the presence of the α_x^A component and other possible cleavage fragments.

The age-related decrease of the thermal stability of bone collagen might be of importance to both the mechanical quality (as discussed above) and the turnover of collagen. A molecular thermal instability that occurs below body temperature could conceivably make the collagen more prone to enzymic attack and thereby facilitate the collagen resorption.

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