Vitreous structure

V. The morphology and thermal stability of vitreous collagen fibers and comparison to articular cartilage (type II) collagen

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Vitreous samples from bovine, ovine, human, canine, and lapine unfrozen eyes were found to contain fine fibrils which displayed no clearly discernible banding pattern when negatively stained with either phosphotungstic acid (pH 8.9) or silicotungstic acid (pH 7.0). The fibrils from the different species were found to be of similar width (10.5 to 12.5 nm) with the exception of the lapine samples which contained narrower fibrils (7 nm). In vitreous samples positively stained with uranyl acetate, cross-striations were observed to occur along the length of the vitreous fibrils of all species studied. These cross-striations represent a repeating band pattern with the major period having an axial repeat distance of 62 nm and containing alternating light and dark bands with 10.5 nm spacings between like bands. This banding was found to closely resemble that of collagen fibrils isolated from articular cartilage. Uranyl acetate–stained segment long-spacing monomers prepared from pepsin-solubilized bovine vitreous and articular cartilage collagens were also found to be very similar in length and banding pattern. The shrinkage temperatures obtained with bundles of vitreous fibrils were comparable for all species studied (59° to 60°), with the exception of the lapine samples which were lower (56° to 57°). Markedly different behavior was observed with thin full-depth slices of articular cartilage. However, the melting temperatures of bovine, ovine, and lapine vitreous and bovine articular cartilage solubilized collagens were found to be the same. These studies further illustrate the similarity in properties of the helical regions of the type II collagens of these two tissues.

Key words: vitreous, collagen, morphology, thermal stability, comparison, species, articular cartilage

The collagenous nature of the fine vitreous fibrils has been demonstrated biochemically,[1-5] morphologically,[2, 5-7, 8] and by x-ray diffraction.[2, 9]

Several morphological studies of the bovine and human vitreous fibrils have been performed.[2, 5-8, 10-12] There has been general agreement that the vitreous fibrils are fine (diameters quoted from 10 to 20 nm) with a narrow range of fibril widths. However, these studies have varied and at times have been apparently contradictory with regard to the fine structure of periodic transverse banding. Maltoltsy et al.[6] reported a faint banding with a period of 64 nm, Schwartz[10] and Smith and Serafini-Fracassini[12] reported a transverse axial banding repeat of 11 to 12

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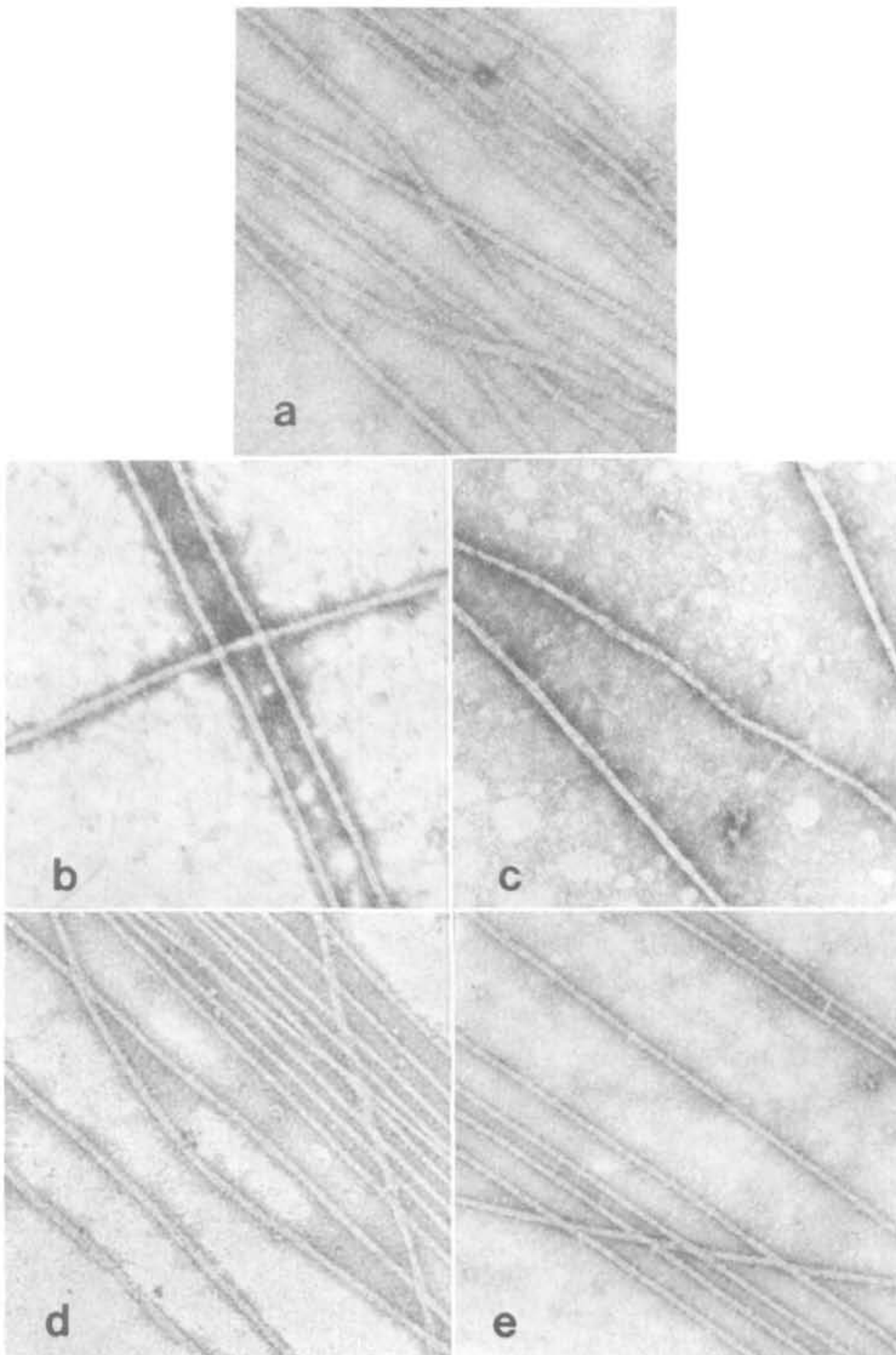

Fig. 1. Morphology of negatively stained (PTA) vitreous fibrils from various species. **a**, Bovine. **b**, Ovine. **c**, Human. **d**, Canine. **e**, Lapine. (×100,000.)

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Table I. Comparison of fibril size and thermal stability

Species	*Average fibril diameter (nm)*	*Ts (°C)*
Bovine	10 ± 2	59-60
Ovine	12.5 ± 1	59-60
Human	12.8 ± 1	-ND-
Canine	12.0 ± 2	59-60
Lapine	7 ± 1	56-57

nm, Swann et al.[5] reported an indistinct axial periodicity of 23 nm, and Blackstad and Vegge[1] and Olsen[7] reported no recognizable repeat in native vitreous fibrils. The extent to which these differences are due to variations in tissue preparation and staining procedures has not been resolved, but the general conclusion has been that vitreous collagen does not display a major period equivalent to the 64 nm period observed with vertebrate collagens from other sources. This led to the proposal that the packing arrangements of collagen molecules in vitreous fibrils differed from the "normal" quarter stagger arrangement of other vertebrate collagens.[7]

Comparative studies involving vitreous collagens have been limited. Olsen[7] has compared the morphology of segment long-spacing (SLS) crystallites prepared from bovine vitreous collagen to those prepared from rat tail tendon, and Smith and Serafini-Fracassini[12] compared the banding pattern observed with vitreous fibrils with those published for collagens from other sources. In both cases the vitreous collagen was compared to type I collagens. Subsequently Swann et al.,[3, 5] using amino acid analysis and disc gel electrophoresis, obtained data which indicated that bovine vitreous collagen is composed of α_1, type II polypeptide chains similar to that found in cartilage. The chemical and morphological structure of the insoluble vitreous protein fractions obtained from bovine and lapine samples after guanidine hydrochloride extraction to remove noncollagenous proteins were, however, very different.[3-5] This observation raised the possibility that the organization of the vitreous fibers differed in various species or that there were marked differences in the types/quantities of proteins associated with the collagen.

A property of collagen that has been extensively studied with collagens from other tissues is thermal stability. This property of collagen can be measured with the thermal shrinkage temperature (Ts) of insoluble fibrils and the melting temperature (Tm) of solubilized collagens. Brunish[13] reported that the Ts of residual protein isolated from bovine vitreous was approximately 60°, but comparative studies were not performed.

In the present study, the nature and properties of vitreous fibrils have been further examined by comparison of the properties of vitreous collagens obtained from a number of species (bovine, human, ovine, canine, and lapine) and by comparison of bovine vitreous collagen with type II collagen obtained from articular cartilage. Comparisons have been made on the basis of morphology and thermal stability of both intact fibrils and pepsin-solubilized collagens.

Materials and methods

Morphological studies. Bovine, canine, lapine, and ovine adult eyes were obtained immediately after death and thereafter maintained at 4°. Unfrozen adult human eyes were obtained from the New England Eye Bank.

After equatorial section of the globe, the posterior vitreous gel was removed with forceps and washed in water. A drop of the vitreous sample was dispersed in water by injection through a syringe fitted with a 15-gauge needle, and a drop of the dispersed vitreous was placed on a grid. Excess moisture was removed with a piece of filter paper.

For negative staining, a drop of 1% (v/w) phosphotungstic acid (PTA) in H_2O, pH adjusted to 8.9 with NaOH, was added to the grid and removed after 10 sec by blotting on filter paper. The grid was then air-dried.

For positive staining, the grids were floated on a droplet of saturated uranyl acetate, in H_2O, for 10 to 15 min. The grid was then washed repeatedly with H_2O and air-dried.

Bovine articular cartilage was obtained by careful dissection of the cartilage from the knee joints of adult animals. The cartilage was then diced into small pieces which were repeatedly (three times)

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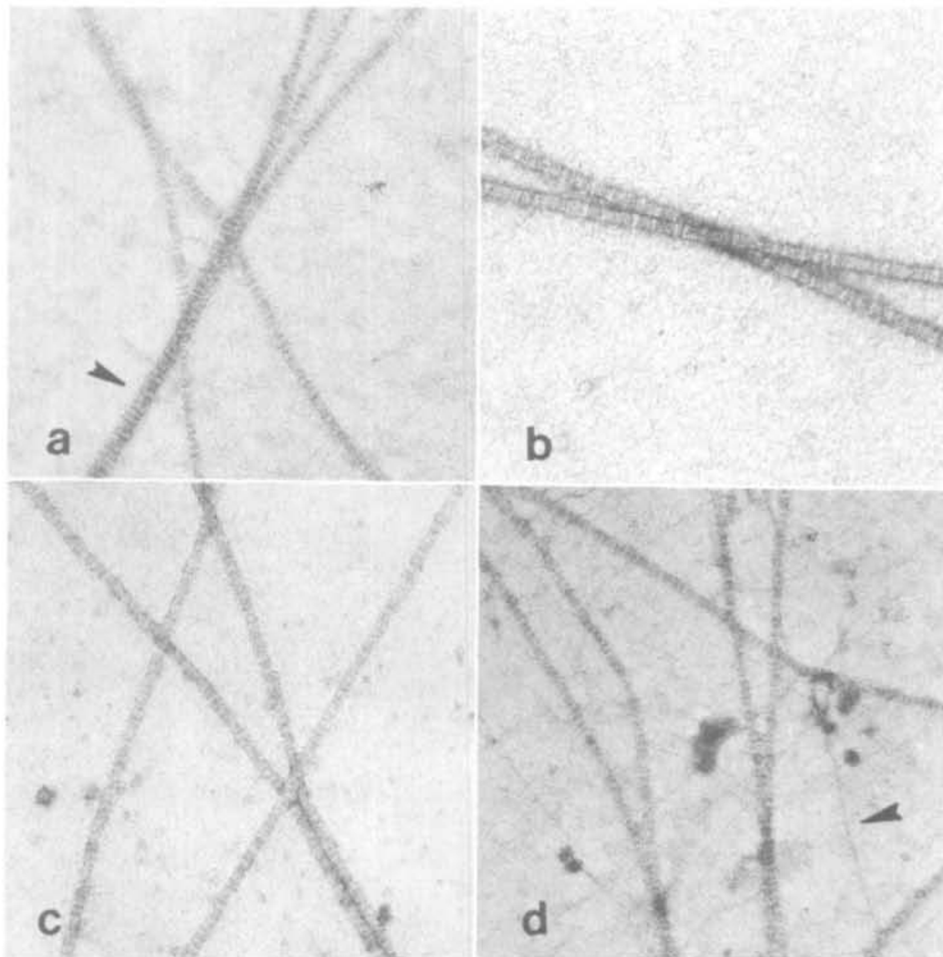

Fig. 2. Morphology of positively stained (uranyl acetate) vitreous fibrils from different species. **a**, Bovine. **b**, Ovine. **c**, Human. **d**, Lapine. (×100,000.)

washed with water at 4° and then lyophilized. The lyophilized cartilage was subsequently milled in a Wiley intermediate mill.

For electron microscopic examination, approximately 0.10 gm of the powdered cartilage material was suspended in 50 ml of buffer (0.15M NaCl, 10 mM phosphate, pH 7.2) and allowed to stand for 2 hr at 4°. The suspension was poured into a measuring cylinder, the large particles were allowed to settle (5 min), and the upper portion of the suspension was decanted off. A drop of the decanted suspension was prepared for electron microscopic examination with essentially the same procedures as those described for the vitreous fibrils.

SLS aggregates were prepared by dialysis of solutions of pepsin-solubilized collagens (0.5 mg/ml in 0.1M acetic acid) against 0.4% ATP, pH 2.8, at 4°. The SLS fragments of collagen were prepared for electron microscopic examination with essentially the same procedure as those described for the vitreous fibrils. Samples were examined with a Phillips Model 300 electron microscope.

Thermal stability studies. Bovine vitreous samples were obtained as described above. The combined vitreous samples were then dispersed by injection through a syringe fitted with a 15-gauge needle. The insoluble "residual" proteins and the soluble constituents were separated by centrifugation at 125,000 × g for 60 min at 4°. The insoluble protein pellets obtained were washed, resuspended in water, recentrifuged (×2), and then lyophilized.

Frozen, adult ovine, canine, and lapine eyes

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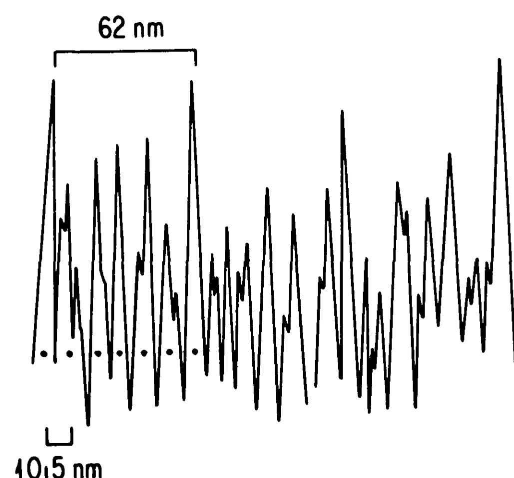

Fig. 3. Densitometer tracing of electron microscope negative of positively stained bovine vitreous fibrils (tracing of Fig. 2, a).

were purchased from Pel-Freeze Biologicals, Inc., Rogers, Ark. The eyes were kept frozen until after dissection. The sclera, choroid, and retina were cut away, and the vitreous was then separated from the lens and anterior tissues. The vitreous, still frozen, was scraped gently to remove any adhering tissue and rinsed in distilled water. The combined vitreous samples were then melted and subsequently treated as described for the bovine vitreous samples.

Pepsin-solubilized collagens were prepared by suspending lyophilized samples of vitreous residue or milled articular cartilage in 0.5M acetic acid at 4° to which was added pepsin (10% w/w of the sample). Digestion was allowed to proceed for 6 days at 4°. The insoluble residue obtained after centrifugation (90,000 × g, 30 min at 4°) was discarded, and the solubilized collagen was precipitated by the addition of sodium chloride (20% w/v) to the supernatant. The precipitated collagen was dissolved in 0.5M acetic acid, reprecipitated with 20% (w/v) NaCl, dissolved in 0.1M acetic acid, dialyzed against this solvent, and then lyophilized.

Ts was determined with the following procedure. Samples of lyophilized intact fibrils were teased out into fine threads (0.5 to 1.0 cm) Each thread was then suspended in buffer (0.15M NaCl, 0.01M phosphate, pH 7.2) and allowed to soak for 30 min at room temperature. The temperature was then raised at a rate of 0.5°/min. The sample was viewed through a telescope against a black background. The temperature of incipient length contraction was taken as the Ts.

The thermal stability of the pepsin-solubilized collagens were compared with viscosity/temperature curves. Viscosities were measured in Cannon-Manning semimicro viscometers (size 75). The pepsin-solubilized collagen samples were dissolved in 0.1M acetic acid at a concentration of 0.5 mg/ml. A 1 ml volume of solution was added to a viscometer prewarmed to 30°, and the sample was allowed to equilibrate for 20 min at this temperature. The temperature was raised in a stepwise manner (1.5° to 2° steps). The viscosity was measured at both 10 and 15 min after each rise in temperature. Thermistor measurements showed that thermal equilibrium within the viscometer was achieved within 5 min.

Results

Morphologic examination. Samples of vitreous from all species studied (bovine, ovine, human, lapine, canine), negatively stained with PTA (pH 8.9), were found to contain fine fibril structures (Fig. 1, *a* to *e*). Samples of vitreous applied directly to the grid showed considerable background staining which could be eliminated or considerably reduced by prior centrifugation of the vitreous and resuspension of the precipitate in saline or by washing the vitreous sample on the grid with saline or H_2O prior to staining.

In all cases studied, the fibrils observed were very fine in (diameter 7 to 12 nm) and displayed no clearly discernible banding pattern when negatively stained with either PTA (pH 8.9) or silicontungstic acid (pH 7). Variations in staining density along the length of the fibrils were observed, but there was insufficient contrast to evaluate whether these variations represented a periodic banding structure. No gross differences in the appearance of the fibrils from the various species were apparent, and they appeared to be typical of the type of fibrils previously described in bovine vitreous.[6, 7]

The diameters of the fibrils present in the vitreous samples from the different species were found to be similar, with the exception of the lapine sample which contained narrower fibers (Table I). The fibril diameters given in Table I were obtained with samples negatively stained with PTA and were calculated with the microscope magnification factors supplied by the manufacturer.

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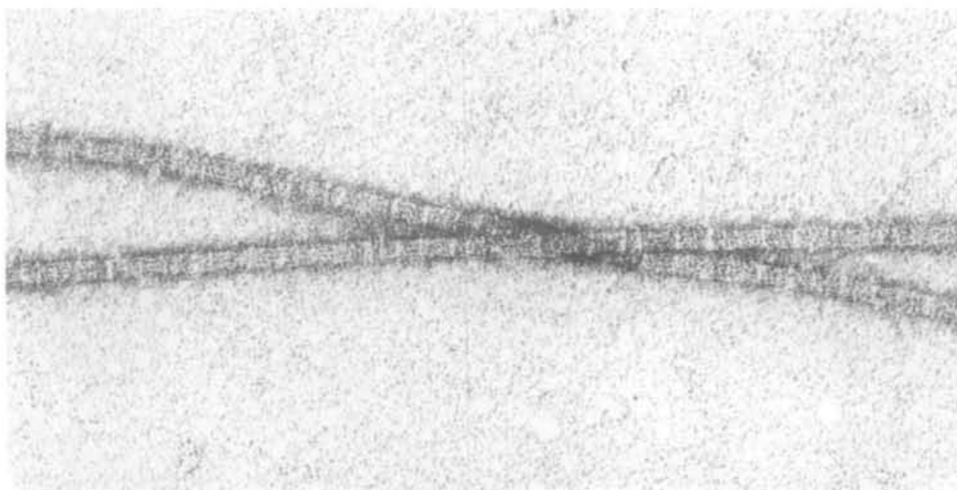

Fig. 4. Enlargement of positively stained ovine vitreous fibrils. (×168,000.)

Fig. 2, *a* to *d*, is an example of bovine, ovine, lapine, and human vitreous fibrils positively stained with saturated aqueous uranyl acetate. Considerably more detail within the fibrils was seen with the uranyl acetate staining. Cross-striations of the fibrils were clearly visible, and considerable fine structure was discernible. Again, no gross differences between the appearance of the fibrils from the various species were apparent.

Detailed investigation of the cross-striation pattern was made difficult by the lack of contrast obtained with the uranyl acetate staining. However, with microdensitometer tracings of electron microscope negatives, a major period having an axial repeat distance of approximately 62 nm could be identified (Fig. 3). The fine structure observed within the major 62 nm periodic repeat is consistent with previous reports of striations with axial periodicities of 64 nm,[6] 10 to 12 nm,[10, 12] and 23 nm[5] occurring along the fine vitreous fibrils (Fig. 3).

It would seem from Fig. 4 (a higher magnification of Fig. 2, *b*) that it was possible to resolve the "microfibrillar" structure of the untreated vitreous fibrils with the use of uranyl acetate. The most common number of microfibrils counted per 10 nm fibrils was 5. It was evident that in this preparation the uranyl acetate acted both as a negative and positive stain. It can be seen in Fig. 2, *d* (arrow) that the lapine vitreous contained, in addition to the 7 nm fibrils, extremely fine fibrillar components. The chemical nature of and the relationship of these components to the larger collagen fibrils was not known.

Lateral aggregates of the 10 nm fibrils with the cross-striation patterns of the individual fibrils in register were also observed (e.g., arrow in Fig. 2, *a*). The most commonly observed larger fibril contained three 10 nm fibrils. A similar observation was made by Smith and Serafini.[12]

The fine structure observed with the 62 nm periodic repeat of the vitreous fibrils appeared to be very similar to that observed with type II collagen fibrils obtained from articular cartilage (Fig. 5). The banding pattern of reconstituted cartilage collagen fibrils has been discussed in detail by Bruns.[14]

SLS dimer formed by the dialysis of pepsin-solubilized ovine vitreous collagen against 0.4% ATP and negatively stained with PTA is shown in Fig. 6. The length of the dimer (485 nm) and extent of overlap (124 nm) were similar to those reported for SLS dimers formed from bovine vitreous collagen.[5, 7] The dimensions of SLS dimers of bovine vitreous and bovine articular cartilage formed under same conditions were found to be similar to those of the ovine sample. Little fine structure of the banding pattern of the SLS forms of collagen was resolved with negative staining. The

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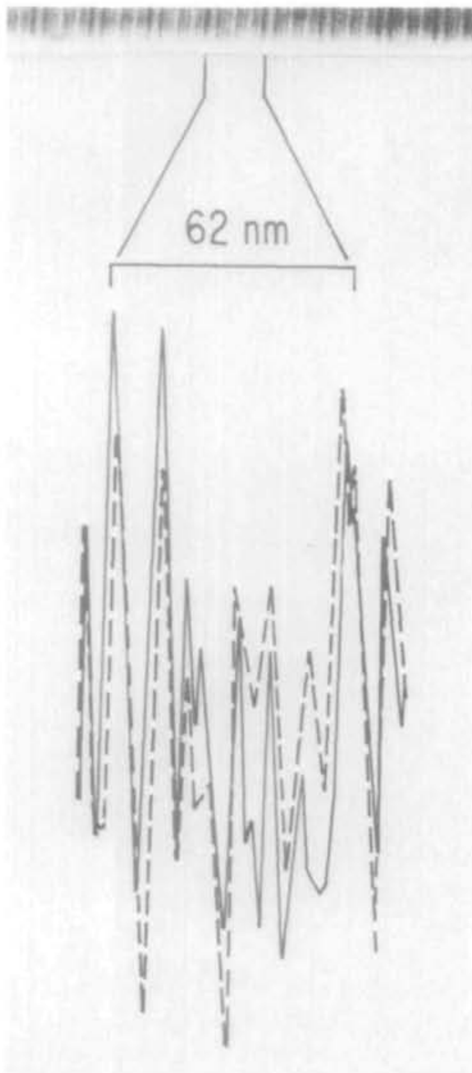

Fig. 5. Comparison of the densitometer tracings obtained with samples of bovine vitreous (solid line) and articular cartilage (broken line) fibrils positively stained with uranyl acetate. Tracings were obtained from the bovine vitreous sample shown in Fig. 2, a, and the uranyl acetate–stained articular cartilage fibril shown in Fig. 5.

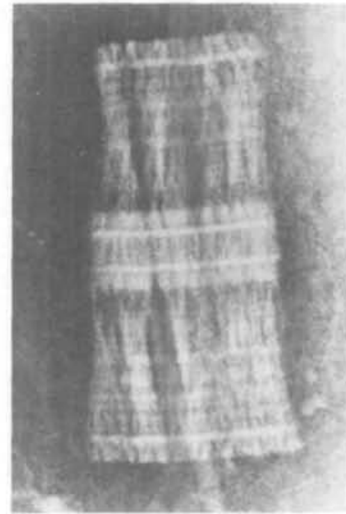

Fig. 6. SLS dimer prepared from pepsin-solubilized ovine vitreous collagen negatively stained with PTA. (×120,000.)

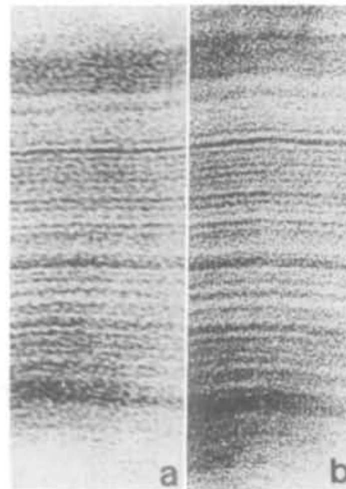

Fig. 7. Comparison of the SLS monomers prepared from bovine articular cartilage (a) and vitreous (b) pepsin-solubilized collagens positively stained with uranyl acetate. (×225,000.)

banding patterns of the positively stained SLS monomers prepared from pepsin-solubilized bovine vitreous articular cartilage collagen were very similar (Fig. 7).

Thermal stability studies

Intact fibrils. The thermal shrinkage temperatures measured for fibril bundles of bovine, ovine, canine, and lapine vitreous collagens are shown in Table I. The Ts of the collagens present in bovine, ovine, and canine vitreous fibrils were similar, but that present in lapine vitreous fibrils had a lower stability.

Thin (50 mμ) full-depth cartilage slices showed a slow contraction over a wide temperature range. The slices began to contract

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at 62° to 63° and continued to do so up to temperatures in excess of 70°. It was therefore considered inappropriate to attempt to assign a Ts to the collagen fibrils within this tissue.

Pepsin-solubilized collagens. The viscosity/temperature curves obtained with samples of pepsin-solubilized bovine, ovine, canine, and lapine vitreous collagens and bovine articular cartilage collagen each dissolved at 0.5 mg/ml in 0.1M acetic acid were found to be very similar (Fig. 8, *a* and *b*). The curve obtained with acid-soluble calf skin collagen, included for comparison, was also very similar.

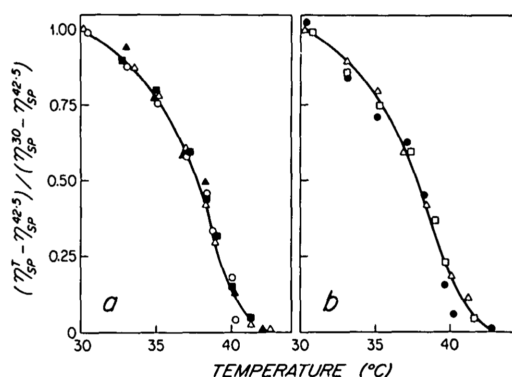

Fig. 8. Viscosity/temperature curves. a, Bovine (△), ovine (■), canine (○), and lapine (▲) pepsin-solubilized vitreous collagens. b, Bovine vitreous (△) and articular cartilage (□) pepsin-solubilized collagens and acid-soluble calf skin collagen (●).

Discussion

With the exception of the lapine samples, the fibril diameters shown in Table I are comparable to the values previously reported for bovine vitreous fibrils by Olsen,[7] Maltoltsky et al,[6] and Smith and Serafini[12] but are considerably smaller than those reported by Swann et al.[5] for guanidine hydrochloride extracted fibrils. It is suggested that the guanidine hydrochloride extraction used previously to purify the collagen caused a swelling of the fibrils. Chemical agents, e.g., acetic acid, are known to be capable of swelling collagen fibrils without significantly altering the band spacing. Furthermore, it is suggested that the very large aggregates described by Swann et al.[3] for rabbit vitreous collagen samples were also a consequence of the prior manipulation of the samples. In the present study, large lateral aggregates of the type previously described[3] were observed in samples which had been lyophilized prior to examination but not in vitreous samples where the vitreous was applied directly to the grids. In terms of morphology and thermal stability, the vitreous collagen fibrils of the various species studied appear to be the same with the exception of the lapine. The lapine vitreous fibrils are different in that they have a narrower fibril diameter and a lower thermal stability than the fibrils of the other species.

The viscosity/temperature curves obtained with the pepsin-solubilized collagens from the various species, including lapine, show that the thermal stabilities of these solubilized collagens are very similar. It would seem therefore that the lower stability of the lapine fibrils is due to a difference in the extent of stabilization resulting from fibril formation, presumably due to differences in the organization and/or composition of the lapine fibrils. Fine fibrillar material was observed in lapine samples (Fig. 2, *d*), and the chemical composition of bovine and lapine insoluble protein fractions differed markedly. The amino acid composition of the pepsinized collagens prepared from these tissues are however similar (refs. 3, 4, and 7 and Swann unpublished observations), which indicated that the observed differences in the structure of the lapine fibers is not due to differences in the type of collagen present.

The similarities in the length and banding patterns of the SLS monomers formed from vitreous and articular cartilage collagens (Fig. 7) indicate that the overall lengths of the helical regions and the distribution of polar amino acids along the length of the helical regions are essentially the same for these two type II collagens.

The viscosity/temperature curves (Fig. 8) obtained with pepsin-solubilized bovine vitreous and articular cartilage collagens show that these two soluble collagens have the same melting temperature. This finding was not unexpected, since these collagens have

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similar amino acid compositions.[3] It was not possible to compare the thermal stabilities of the fibrillar forms of bovine vitreous and articular cartilage collagens with Ts used as the criterion because of the behavior observed with thin cartilage slices. The behavior of the cartilage slices may have been due to the high levels of proteoglycans present in this tissue. However, the Ts's temperatures obtained with the vitreous collagen samples are comparable to the values reported for other collagenous tissues, e.g. tendon.

In summary the collagenous fibrils present in the vitreous appear to be a distinctive form of collagen aggregation in that the fibrils are extremely fine and do not display a 64 nm periodicity when negatively stained with PTA. However, many features of the bovine vitreous collagen are very similar to those of cartilage type II collagen, particularly with regard to the helical regions of these molecules. The similarities include amino acid composition,[3] the types of cyanogen bromide peptides,[15] Tm (Fig. 8), the length of the helical regions,[7] and distribution of polar amino acids along the helical regions. Furthermore, in the present study, the banding pattern observed with positively stained bovine vitreous fibrils was found to closely resemble that exhibited by positively stained articular cartilage fibrils (Fig. 5). This suggests that the organization of the collagen molecules within collagen fibrils of these two tissues are similar.

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