Collagens of the Bovine Vitreous

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The predominant structural components of the vitreous are collagenous fibrils. Prior biochemical analyses have been limited by incomplete solubilization of the constituent collagen types. The techniques described enable an effective separation and nearly complete solubilization of calf vitreous fibrils. Semiquantitative analysis has led to the detection of types II, V, and IX collagen in a ratio of 69:24:7, respectively. Qualitatively similar ratios were found in vitreous from other mammals, including rabbits, monkeys, and humans. Electron microscopic evidence suggests that type VI collagen may be distributed selectively near the vitreous base. These observations are pertinent to vitreous gel stabilization, aging, and disease. Invest Ophthalmol Vis Sci 32:1540–1550, 1991

Clinical observations have shown that alterations in the vitreous accompany various retinal diseases.1 The critical role of the vitreous in the development of rhegmatogenous retinal detachment has been well documented. In addition, the vitreous is thought to be a factor in conditions such as cystoid macular edema, lamellar foveal degeneration, proliferative vitreoretinopathy, diabetic retinopathy, and retinopathy of prematurity. Also, various hereditary vitreoretinopathies are accompanied by marked vitreous changes such as syneresis and membrane formation. However, the precipitating or accompanying biochemical alterations in the vitreous in many of these conditions are largely unknown. An understanding of the factors responsible for the mechanisms of homeostasis in normal vitreous would be helpful in establishing criteria to which we might establish corollaries of pathologic conditions.

Several studies have shown that the major structural component of the vitreous body is nonbranching collagenous fibrils with diameters reported from 7–28 nm.2,3 Various banding patterns or none have been observed in positively or negatively stained vitreous fibrils by transmission electron microscopy. A second, less abundant, filamentous structure was originally noted by Matoltsy et al4 and recently reexamined by Wright and Mayne.5 This filament consists of a succession of linked beads (diameter, approximately 20 nm) with a spacing varying from 35–85 nm. Our studies on this “beaded filament” or chain will be reported separately.

Covalent bonds apparently link the collagen molecules that form the collagen fibrils because they are insoluble in all the usual protein solvents, but they have been dissolved (albeit incompletely) after digestion with pepsin at 4°C. Numerous biochemical studies have identified type II collagen, a homotrimer of α1(II) chains, as the major component of vitreous collagens.6–9 More recent reports have noted additional components of the pepsin-solubilized vitreous collagen. Ayad and colleagues10,11 detected “cartilage-phosphate-soluble” (C-PS) collagens, C-PS 1 and C-PS 2, in bovine vitreous. These components were later attributed to the pepsin-resistant fragments of type IX collagen (high and low molecular weight, respectively).12 Additionally, Ayad and Weiss11 reported the 1α 2α 3α collagen (type XI) in their preparations of pepsin-solubilized vitreous collagen, but Eyre and Wu13 identified type V collagen in bovine vitreous, not type XI.

We report a procedure for the effective separation of collagenous fibrils from other vitreous components. Almost complete solubilization of these fibrils was achieved. Analyses of such solutions derived from bovine vitreous confirmed the presence of types II, V, and IX collagen, and we measured semiquantitatively the ratios of these components. Against this more complete analysis of the normal vitreous, it may be profitable to compare the analyses of aged and diseased vitreous. We also undertook preliminary analyses of rabbit, monkey, and human vitreous.

Materials and Methods

Calf and cow eyes were obtained from a local abattoir and maintained on ice until their dissection 4–6
hr post mortem. A 360° circumferential incision was made through sclera, choroid, and retina into the vitreous cavity. The anterior segment with adherent vitreous was lifted from the posterior segment which separated cleanly and was discarded. The total vitreous was then gently dissected from the adherent ciliary body, zonules, and lens; separation was facilitated by using a club-shaped retinal spatula. To obtain vitreous samples from distinct anatomic regions for their separate analysis, the anterior segment with the globe was discarded. The total vitreous was immersed for approximately 20 sec in 0.2 M acetic acid with 0.05% pepsin (Worthington, Malvern, PA). This suspension was then carefully layered on top of a 40-ml nitrocellulose tube and centrifuged at 100,000 × g for 16 hr in a Beckman rotor TiS28. When the tube was examined under lateral illumination, two clearly resolved bands could be seen below a broad turbid zone. The opaque, white middle band appeared highly viscous and often contained readily discernible coarse filaments extending from it in centrifugal and centripetal directions (dragged out of the band by floating or sinking, adherent tissue). This band was recovered by aspiration, dialyzed against 0.05 M ammonium bicarbonate for electron microscopy or against water, and then against 0.2 M acetic acid for pepsin treatment. Below the band of fibrils a less prominent band was observed that contained the beaded filaments. Studies on that component will be described elsewhere.

Similar preparations were made from young rabbit, monkey (macaque), and human eyes. The latter were obtained through the New England Eye Bank. The monkey eyes were provided by Dr. M. Tigges (Yerkes Regional Primate Center, Emory University). The New Zealand white rabbits were bred at our institute and killed with an overdose of intravenous pentobarbital.

The investigation using animals conformed to the ARVO Resolution on the Use of Animals in Research.

Collagen Isolation

Our method for collagen isolation is summarized in Figure 1. Whole vitreous was mixed with protease inhibitors (5 mM ethylenediaminetetraacetic acid [EDTA], 2 mM benzamidine hydrochloride, and 1 mM p-hydroxy-mercuribenzoate) and homogenized with 25 strokes in a 30-ml Dounce tissue homogenizer (Wheaton, Millville, NJ). The dispersion was centrifuged at 100,000 × g for 45 min in a model L5-50 ultracentrifuge (Beckman, Palo Alto, CA). The insoluble residue, which included the collagen fibrils with associated proteoglycans, was then resuspended in 20 volumes of the solvent ED (1 M ethylene diamine hydrochloride, pH 8.0; Fluka, Ronkonkoma, NY) containing the added enzyme inhibitors. This preparation was dispersed and sheared with 25 strokes in a 1-ml Dounce tissue homogenizer, and gently agitated overnight. The resulting suspension was centrifuged at 50,000 × g for 30 min, after which the insoluble residue was washed twice in water, and the washes were combined with the ED-soluble fraction. This fraction was then dialyzed against water for 24 hr and lyophilized. The ED-insoluble material was resuspended and homogenized extensively in 0.2 M acetic acid with 0.05% pepsin (Worthington, Malvern, PA). The fibril suspension was then gently agitated overnight. High-speed centrifugation of this digest yielded an insoluble residue accounting for 1–5% of the total collagen from calf vitreous.

A purer preparation of the collagenous vitreous fibrils was obtained by zone sedimentation. Fresh calf vitreous was mixed with protease inhibitors and with 1:9 volumes of 3 M ED and homogenized by repeated, successive passages through 18-, 21-, 23-, and 25-gauge hypodermic needles. Fifteen milliliters of this suspension was then carefully layered on top of a continuous cesium chloride gradient (2.0–3.3 M) containing 5 mM EDTA in a 40-ml nitrocellulose tube and centrifuged at 100,000 × g for 16 hr in a Beckman rotor TiS28. The supernatant was then dialyzed against 0.05 M ammonium bicarbonate for electron microscopy or against water, and then against 0.2 M acetic acid for pepsin treatment. Below the band of fibrils a less prominent band was observed that contained the beaded filaments. Studies on that component will be described elsewhere.

Portions of the pepsin-solubilized material from either type of collagen fibril preparations were fractionated by selective salt precipitation. Collagens were first precipitated by dialysis to 0.9 M NaCl in 0.5 M acetic acid, pH 3.0 (they are referred to as fraction 1 in this text). The supernatant was then dialyzed to 2.0 M NaCl to give a second fraction (fraction 2). An alternative fractionation scheme involved successive precipitations at 0.9, 1.2, and 2.0 M NaCl in 0.5 M acetic acid. The resulting fractions were then analyzed with the following techniques.

Gel Electrophoresis

The samples were suspended in sodium dodecyl sulfate-containing buffer, heated briefly, and then analyzed on polyacrylamide gels (SDS-PAGE, mini protein II; Bio-Rad, Richmond, CA) as previously described. The ratios of the various collagen chains identified on the gels were obtained by measurement of absorbance of the Coomassie blue-stained gels using a Joyce-Loebl scanning densitometer (Dusseldorf, West Germany) and by eluting the Coomassie blue from the excised bands with 25% pyridine and measuring the absorbance at 600 nm on a Shimadzu spectrophotometer (Kyoto, Japan). Some gels were silver stained to enhance detection of minor components.
Additionally, the identification of several minor collagen components was aided by separating the mixture on 6% SDS-PAGE, transferring the briefly stained bands individually to a 6–15% gradient SDS-PAGE, and rerunning after reduction with mercaptoethanol. Other separated bands were subjected to cyanogen bromide (CNBr) digestion and then rerun to yield a peptide map.\(^\text{18}\)

**Bacterial Collagenase Digestion**

The collagenous nature of the bands observed on SDS-PAGE was demonstrated by their susceptibility to highly purified bacterial collagenase, type III (Advance Biofactures, Lynbrook, NY). Samples were dialyzed to 0.05 M CaCl\(_2\), 0.1 M NaCl, 0.1 M Tris, pH 7.6 and incubated overnight with enzyme at 20°C. They were then dialyzed to 0.05 M acetic acid.

**Electron Microscopy of Segment-Long-Spacing (SLS) Crystallites**

Pepsin-solubilized collagen preparations in acid solution were dialyzed to 0.4% adenosine triphosphate in 0.05 M acetic acid at 4°C, and the SLS precipitates were applied to a cold, carbon-filmed 400-mesh grid. Excess liquid was drained, and the specimens were negatively stained with cold, 1% phosphotungstic acid, pH 4.2, drained, and examined on a Philips EM 300 microscope (Mount Vernon, NY). The banding patterns of the SLS crystallites were compared with segments prepared from purified collagen preparations. For this purpose, types I, II, III, V, and XI collagens were fractionated from acid extracts or pepsin digests of tendon, cartilage, fetal skin, cornea, and cartilage, respectively.

**Rotary Shadowing**

After dialysis against 0.05 M ammonium bicarbonate, the samples (with glycerol to 20% by volume) were sprayed onto freshly cleaved mica sheets, rotary shadowed with platinum at 3–10° angle, and then covered with carbon evaporated from a vertical position.\(^\text{19}\) The carbon films were stripped on water and picked up on 400-mesh copper grids for electron microscopy.

**Serologic Tests**

Pepsin-solubilized calf vitreous collagens were assayed for type VI collagen using a mouse monoclonal antibody directed against a heat-sensitive epitope of the molecule (prepared by Dr. Eva Engvall and a gift from Dr. Charles Cintron). Briefly, aliquots of collagen solutions without and with prior heating to 70°C

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**Fig. 1. Algorithm for collagen isolation from vitreous.**

A  
Homogenized Vitreous  
-centrifuged 45 min, 100,000g  

Insoluble Vitreous (primarily collagenous fibrils)  

Resuspend in 1M ethylene diamine; homogenize; agitate overnight  
-centrifuge 30 min, 50,000g  

Ethylene Diamine insoluble (presumptive collagen fibrils)  

B  
Vitreous  
-suspend in 0.3M Ethylene diamine; homogenize through hypodermic needles  

Isopycnic Centrifugation  
16 hours, 100,000g  

Collagen Fibril Band (presumptive)  

Electron Microscopy  

Resuspend in 0.2M Acetic Acid with 0.05% pepsin; homogenize; agitate overnight  
-centrifuge 30 min, 50,000g  

Soluble Collagens  

Salt Fractionation
were blotted on nitrocellulose paper. After blocking, binding of the primary antibody was detected by the peroxidase-labeled second antibody coupled to the reaction products of diaminobenzidine.

Results

The particulate components of the homogenized vitreous can be centrifuged down to a compact pellet to eliminate most of the soluble proteins and compact the fibrils for analysis. However, the pellet must include the beaded filaments and cellular debris. A better preparation of fibrils was obtained from zone sedimentation. This technique yielded a zone of cellular debris and uncharacterized particulates floating above a band of collagenous fibrils that in turn was well separated from the band of beaded filaments.

Examination of the soluble components of the vitreous by SDS-PAGE yielded no detectable collagen or procollagen chains (data not presented). From 30 ml of calf vitreous we obtained about 2–5 mg dry fibrils. Adult bovine vitreous yielded 4–6 mg from a similar volume.

In the electron microscope the fibrils appeared as unbranched, relatively stiff cylinders with a diameter about 20 nm (Fig. 2). The lengths of the fibrils could not be defined because even after shearing, their ends were observed infrequently in any microscopic field. Because of regional variations in fibril content, volumes from central, cortical, anterior, and posterior vitreous were examined by electron microscopy. The predominant vitreous fibril seemed similar in every region of the vitreous. "Long-spacing fibrils" were observed (Fig. 3) but only from the anterior vitreous ad-

Fig. 2. Four electron micrographs of calf vitreous fibrils in negative stain (A) or rotary shadowed from 0.05 M ammonium bicarbonate-glycerol (B, D) or from 0.05 M acetic acid (C). The apparent diameters of the fibrils are approximately 20 nm in (A) and 28 nm in (B) and (D), but both show a transverse banding with a banding averaging 15 nm and indications of a 70–75 nm periodicity (A). The component filaments are seen in acidified preparations (C) (see also Olsen).
Fig. 3. Negatively stained dispersed calf vitreous from the area adjacent to the peripheral retina and pars plana. Three short assemblies with paired cross-striations and a period of 110-140 nm can be seen (arrowheads). Similar structures have been called "long spacing fibrils" and these may be type VI collagen fibrils.

Fig. 4. Four tracks on a 6%-15% polyacrylamide-SDS gel with unreduced samples of a pepsin digest of calf vitreous collagen. The samples had been incubated with 0, 1, 3, and 10 μg pure collagenase before electrophoresis. All the bands in track 0 are vulnerable to collagenase. The major band in that track, α1(II), is marked with an asterisk.

Jacent to the ora serrata and peripheral retina (ie, the vitreous base). Such structures resemble fibrils of type VI collagen.21

Collagen Composition

To analyze the collagens that comprise the fibrils, we first treated with ED. While apparently extracting essentially no soluble collagen, this treatment changed the aggregating tendencies of the fibrils and subsequently the solubilization of calf fibrils by pepsin was 95-99% complete. Solubilization by pepsin was significantly less complete if the fibrils had been lyophilized or if the solutions were not salt free. Extraction with 4 M guanidine HCl before pepsin digestion also was tried, but it did not improve solubilization of collagen fibrils as effectively as did ED.

The proteins in the pepsin digest were examined by SDS-PAGE. Multiple bands were observed, but all of them were collagenous by the criterion that they could be digested by purified bacterial collagenase (Fig. 4). A progressive increase in the concentration of bacterial collagenase in such digests caused a commensurate elimination of all the observed bands. We also applied samples of the small, pepsin-insoluble residues to SDS-gels. Traces of type II and type V collagen chains were seen, but most of the Coomassie blue-stained material did not enter the running gel.

To resolve the complex pattern of bands observed on SDS-PAGE and identify the collagens, two fractions were precipitated from the total pepsin-solubilized vitreous collagens in 0.5 M acetic acid by dialyzing to solutions initially with 0.9 M NaCl in 0.5 M acetic acid (fraction 1) and then with 2.0 M NaCl in 0.5 M acetic acid (fraction 2). The components in fraction 1 were unchanged by reduction, but on reduction of fraction 2, the pattern of stained bands changed dramatically (Fig. 5). The components in fraction 1 could be resolved further by redissolving in 0.5 M acetic acid and dialyzing to 0.9 M NaCl in 0.5 M acetic acid. Most of the material precipitating showed on SDS-PAGE a single α-chain with the mobility of α1 (II). The collagen remaining dissolved yielded two α-chains with the mobilities of α1 (V) and α2 (V) on electrophoresis (not shown).

Another pepsin digest of calf vitreous was used to identify the vitreous components in fraction 2. The contributions that each of the higher molecular weight, unreduced chains made to the lower molecul-
To establish the difference between the collagens of vitreous and cartilage, a pepsin digest from calf hyaline cartilage was fractionated in acetic acid/NaCl like the vitreous, and its fraction 2 underwent electrophoresis (Fig. 7). A difference was noted among those bands whose mobilities are unchanged by reduction. Although the presumptive $\alpha_1$ (V) chain from vitreous matches that of cartilage $\alpha_1$ (XI), only one additional alpha-length chain was noted in vitreous ($\alpha_2$ (V)) which migrated to an area intermediate between $\alpha_2$ and $\alpha_3$ (XI) of cartilage.

**Electron Microscopy**

To check the tentative identifications, SLS crystallites were prepared from fractions 1 and 2 from vitreous and cartilage. Electron micrographs of the SLS were compared with those from purified preparations of types I, II, III, V, and XI bovine collagens. These experiments showed both type II and type V collagens in vitreous fraction 1. In fraction 2 we found type V and SLS fragments 63% and 36% of the length of type II segments, and symmetric dimers of fragments 37% and 50% of that length (Fig. 8). Similar segments to some of these have been ascribed to type IX molecules cleaved by pepsin across the triple helix. In fraction 2 from calf cartilage, we observed SLS crystallites identical to corneal type V but no unusual crystallites that might be attributed to type XI.

**Peptide Mapping**

The CNBr peptides of the $\alpha$-chains in fractions 1 and 2 were detected by silver staining after SDS polyacrylamide electrophoresis. Fig. 6. A gradient SDS-polyacrylamide gel with: track 1. sample of fraction 2 from a pepsin digest of calf vitreous fibrils. Gel slices corresponding to bands numbered 3–7 and 9 were cut from a similar gel, reduced with mercaptoethanol, placed into the sample slots of this gel, and rerun on tracks 3–7 and 9. Track 8 is the same sample as 1 after reduction. Because the samples in the gel slices were retarded by the gel itself, they all lag slightly behind the equivalent bands in tracks 1 or 8. The bands 3 and 4 include $\alpha_1$ (V) and $\alpha_2$ (V), respectively.

Fig. 5. Two 6%–15% SDS-polyacrylamide gradient gels with successive samples of fraction 1 (1.3, 2.6, and 3.9 ml) and fraction 2 (5, 10, and 15 ml) from a pepsin digest of calf vitreous fibrils. The last track contained type I collagen; the $\alpha_1$ (I) and $\alpha_2$ (I) chains are labeled 1 and 2. The type II $\alpha$ chain is marked with an asterisk. The lower gel samples were reduced with mercaptoethanol before electrophoresis. Stain: Coomassie Blue. Traces of pepsin (marked P) appear as two bands after reduction.

lar weight components after reduction with mercaptoethanol were examined (Fig. 6). The three bands (5, 6, and 7), migrating more slowly than $\alpha_1$ (V) each gave rise to a band the size of an $\alpha$-chain in addition to several components in the molecular weight ($M_\text{r}$) range of 60,000 and to some in the $M_\text{r}$ 10,000 range. The single band numbered 9, of $M_\text{r}$ 30,000–40,000, was seen before reduction yielded three separate bands of $M_\text{r}$ 5–10,000 after reduction. This latter component has the characteristics of the low molecular weight fragment (LMW) of Type IX collagen. We conclude that fraction 2 contains pepsin-treated type V and type IX collagen.
acrylamide gel electrophoresis (Fig. 9). These patterns were compared with those obtained from pepsin-solubilized electrophoretically separated chains from calf types I, II, and V collagen. A convincing similarity was observed between the presumptive type V maps from vitreous and corneal collagens, and there was a clear resemblance between the maps of cartilage type II α-chains and those from the vitreous.

**Serologic Tests**

There was considerable similarity between the electrophoretic mobilities of some of the reduced fragments of what we presume was type IX collagen from vitreous and the pepsin-resistant fragments of type VI collagen that we prepared from calf intervertebral disc by the method of Wu et al. Type VI chain fragments might thus be obscured in the electrophoresis of the vitreous digests. To test whether type VI collagen also is present in the vitreous, a type VI-specific monoclonal antibody was used in an attempt to identify this component in vitreous extracts and digests. Weak staining was detected by an enzyme-linked assay on a blot of the pepsin digest, and this reaction was lost after heating the collagen solution before the assay (data not presented). This antibody has been shown to recognize a conformation-specific epitope on the type VI molecule, so the loss of immunoreactivity with denaturation would be consistent with the presence of a very small fraction of type VI collagen in the vitreous.

**Quantitative Analysis**

To estimate the ratios of the identified collagens in the vitreous, carefully measured volumes of fractions 1 and 2 were applied to gels and subjected to electrophoresis. The gels were stained with Coomassie blue and then destained in isopropanol:acetic acid:water (1:1:8) at 4°C and scanned by a densitometer. Then stain was eluted in 25% pyridine from each protein band, and the absorbance was measured at 600 nm. In addition, segments of gel of appropriate area were sliced from lengths of each gel track where no protein bands were present to serve as blanks. In every case (barring difficult locations where two bands overlapped), plots of the absorbance of the eluted dye varied linearly with the volume of sample applied to the gel. The slopes of such plots were used as an averaged measure of the protein in the band. We compared each band in each track in two gels with unreduced proteins and two with mercaptoethanol-reduced proteins; we ran both fractions 1 and 2 from a pepsin digest of zone-centrifuged fibrils and reached the conclusions listed hereafter. The justification for these deductions will be given subsequently.

In fraction 1 the ratios of dye in bands $a_1(V)$, $a_2(V)$, and $a_1(II)$ were 1:6.45 and 1:5.89 and 1:6.19 and 1:6.78 (oxidized and reduced) in two pairs of gels. Thus, the ratios were not significantly changed by reduction, so type IX chains were not present in this fraction. The ratio of types II:V in fraction 1 can then
Fig. 8. SLS crystallites prepared from fraction 2 of a pepsin digest of calf vitreous fibrils. Negative stain 1% phosphotungstic acid, pH 4.3. The large arrow indicates the length of the type V molecule. A and B designate two different, symmetrical SLS crystallites that were frequently observed and that appear to be formed from fragments of type IX collagen chains. The A segments are 31% of the length of the type V crystallite and form head-to-head dimers. The B segments are also dimers of chain fragments 47% the length of type V. Segments substantially shorter than 30% length were also seen consistently.

be determined if the ratio of stain in α1(V):α2(V) is known and if a correction is made for the respective β-components. Calibrating experiments with corneal type V collagen (α1-α2) showed that dye ratio to be 1.8:1. In this way the ratios of the collagens were calculated to be (II:V) 3.34 and 3.43 and 3.58 and 4.02 in this particular fraction 1.

In the tracks with reduced samples of fraction 2, the total absorption of the dye extracted from all the bands was 12% less than that extracted from the gel with unreduced samples. In contrast, the extracted dye from the chains from fraction 1 and from tendon (type I) collagen chains applied as calibrating tracks on each gel were not changed by reduction. This difference suggests that the loss of dye-binding after reduction was significant and may indicate that the smaller chain fragments bind Coomassie blue less strongly than larger chains. Therefore, we could not reliably measure the ratios of type V chains to type IX chains and fragments by dye absorbance. To calculate the contribution of type IX chains we summed the dye in all the bands and subtracted that attributable to type V, i.e., the β and α1 and α2 chains in Fig. 5 (lower), those that did not change on reduction.

As a result of the measurements we found the ratio of types V:IX measured in fraction 2 was 1:1.35 and 1:1.45 in two reduced gels. From the volumes of the two fractions and the total dye in the gels the ratios of types II:V:IX in the calf vitreous were then calculated to be 70.0%;23.1%;6.8% from one gel pair and 67.0%, 25.2% and 6.9% from the other.

Changes Related to Maturation and Aging

The vitreous extracted from more mature cattle was treated as described for the calf vitreous. After zone sedimentation, dialysis to water, and pepsin treatment in 0.2 M acetic acid, approximately 80-

Fig. 9. Pepsin-solubilized bovine types II and V collagens were subjected to SDS-PAGE and the α-chains were cut from the gel, digested with CNBr, and the products were again subjected to electrophoresis and then silver-stained. The CNBr peptides in the adjacent tracks confirm the presence of types II and V collagens in the vitreous. (A) Cornea and vitreous α1(V) peptides on a 10%-22% gradient polyacrylamide gel; (B) cornea and vitreous α2(V); (C) cartilage and vitreous α1(II) on a 6%-15% gel.
90% of the fibrillar collagens were solubilized, and electrophoresis demonstrated that the respective fractions 1 and 2 obtained from such solubilized collagens showed roughly similar ratios of \( \alpha \)-chains and chain fragments to what we observed with the calf samples. However, a substantial fraction of the "solubilized" material was apparently aggregated or crosslinked and migrated into and below the spacer-running gel interface. We therefore can draw no conclusion about age-related changes in the composition of the bovine vitreous even over the limited age span encompassed by animals coming to the commercial market.

Vitreous from Other Mammals

To check the generality of our observations on the bovine vitreous, we examined in a preliminary fashion the vitreous of other animals. Preparations of vitreous fibrils were obtained in small volumes from rabbits, monkeys, and humans. In each of these cases the pepsin digestion of ED-treated vitreous fibrils yielded preparations of solubilized collagen where the distributions of \( \alpha \)-chains and chain fragments were very similar to those observed with the bovine samples. Despite ED treatment, however, not all the fibrils from mature animals could be solubilized by pepsin. We conclude that types II, V, and IX collagens are present probably in similar ratios in the vitreous fibrils of these animals to those in cattle. Beaded filaments were also observed by electron microscopy in each of these preparations.

Discussion

These experiments showed that through zone sedimentation in a cesium chloride gradient the particulate components of the vitreous, including the collaginous fibrils and the beaded filaments, can be separated from the soluble components and from each other to permit their independent analysis. We also observed that homogenization of the vitreous in 0.3–0.5 M ED appeared to extract a very small amount of material, probably proteoglycans, adsorbed to the surface of the fibrils. This treatment increased the tendency of the fibrils to aggregate in low ionic strength solutions and improved their resolution by isopycnic centrifugation. On subsequent treatment with pepsin, we achieved almost complete solubilization of the component proteins, virtually all of which appeared to be collagens types II, V, and IX. Only traces of collagen chains were detectable in the very small pepsin-insoluble residues. Because the solubilization was essentially complete, we attempted a quantitative analysis of these collagens, but because the total separation of the collagen types was not achieved, we analyzed the ratios of the collagens in the fractions obtained from these preparations by densitometry of the stained bands separated by electrophoresis on PAGs. We found that analysis by eluting the dye from the gel was more satisfactory than attempting direct densitometry of the gel. However, two practical problems remain. The dye-absorbance measured on fraction 1 (types II and V) did not differ whether the samples were reduced with mercaptoethanol or not before electrophoresis. However, in fraction 2 the total absorbance of the stained bands was observed to diminish after reduction, suggesting that the dye eluted from the bands may not be proportional to the weight of the bands. Furthermore, we noted that the ratio of the dye on the \( \alpha_1 \) to \( \alpha_2 \) chains in type V collagen was not the expected 2:1, but 1.8:1. This observation presumably indicates either that chains even of similar length do not bind similar amounts of Coomassie blue under the conditions we used, that the type V collagen in the vitreous may not be exclusively \( \alpha_1(\alpha_1)_2 \alpha_2 \), or that it may contain chains other than \( \alpha_1(\alpha_1)_2(\alpha_2) \), as has recently been shown to be the case in a type V fraction from bone.

Despite these limitations, we achieved results of reasonable consistency which suggest that in the calf fibrils the well-recognized type II collagen may comprise less than 70% of the total collagens, type V may comprise 25%, and type IX at least 5%. Traces of type VI may also be present. Some uncertainty in the exact quantitation of type IX remains. The distribution of bands migrating above \( \alpha_1(\alpha_1)_2 \alpha_2 \) in the electrophoresis gel of fractions 2 from different pepsin digests (Figs. 5–7) showed some variability. From one to three bands were noted in this position. When these bands were excised, reduced with mercaptoethanol or not before electrophoresis again, they liberated chain fragments (of type IX) in addition to \( \alpha \)-length chains. The pepsin digests showing fewer bands above \( \alpha_1(\alpha_1)_2 \) before reduction contributed, after reduction, proportionately more to the type IX fragments (compare Figs. 6, 7 to Fig. 5). Furuto et al. examined type IX collagens from rat tissue; they observed that pepsin digestion produced progressively smaller fragments. It is likely that the variability noted in the higher molecular weight components in our preparations reflects the completeness of pepsin digestion, such that the digestion in Figure 5 progressed further than those in Figures 6 and 7.

The complex composition of these vitreous fibrils raises some interesting considerations. The triple helical fibril of type I collagen is reported to have a diameter of the order of 1.3–1.4 nm, so a vitreous fibril, if it is cylindric, probably is built from no more than 100 colinear filaments. Electron microscopy of acid-treated or enzyme-treated fibrils suggests fewer, perhaps 15–20 (Fig. 2). The uniformity of the morphol-
ology of the fibrils and their uniform banding density in the sedimentation experiments gives no indication that the collagenous fibrils are heterogeneous (beyond the very small number of unusual fibrils noted in the vitreous base). We conclude that the different collagen types are probably distributed along each fibril.

Type IX collagen in cartilage has recently been shown to be covalently bound to the surface of type II fibrils through a hydroxyproline ring crosslink.28,29 Our observations of regularly occurring protrusions from the surface of vitreous fibrils seen in negative stain are compatible with the regular insertion of type IX molecules which, with a covalently bound chondroitin sulfate chain,30 probably promotes cohesion between the fibrils and hyaluronic acid or other ground substance components in the vitreous gel. Type V may coat, or co-aggregate with, the type II molecules to produce the continuous filament structure in the fibril. The co-distribution of types I and V collagen in the same fibril has been reported in the cornea.31 Although no such relationship has been documented between types II and V collagen, the uniformity of the vitreous fibril ultrastructure supports this concept. The resulting assembly of three collagen types forms a fibril which does not appear to be repaired or turned over and yet provides a gel of substantial strength and stability over many years in the eyes of the longer-lived vertebrates. The organization and modes of interaction of these collagens provide a challenge for further investigation. It is not known if the beaded filament plays a structural role in the vitreous.

We were unable to confirm the presence of 1α, 2α, 3α (type XI) collagen in bovine vitreous as reported by Ayad and Weiss.13 On SDS-PAGE, parallel tracks with 1α 2α 3α of cartilage and fraction 2 from vitreous showed clear differences (Fig. 7). However, we should note that the SLS crystallites from fractions 2 of both vitreous and cartilage matched those from type V collagen from cornea. Therefore, either cartilage contains both types V and XI (and type XI crystallites have not been observed or identified) or the SLS from types V and XI are undistinguishable. It has already been noted that α3(XI) differs little if at all from α2(V).13

Although our preparations of vitreous collagens showed slight reactivity to anti-type VI antibodies, we have no conclusive evidence that type VI collagen is distributed in calf vitreous. The similar mobilities of reduced type IX high molecular weight fragments and the pepsin-resistant fragments of type VI on SDS-PAGE precludes distinguishing them on electrophoretic evidence alone. However, the unusual fibrils noted in the vitreous base area were similar to those identified as type VI fibrils.21 For this reason we have not considered type VI collagen in our quantitative measurements. Since the electrophoretic profiles of the pepsin-solubilized collagens from the various regions surveyed were similar despite the apparent sequestration of long-spacing fibrils to the vitreous base, type VI collagen is unlikely to represent a quantitatively significant fraction.

In summary, we purified and almost complete solubilized calf vitreous fibrils and did a semiquantitative analysis. The roles of types V and IX collagen, in addition to type II collagen, in the functional organization of the vitreous have been discussed. Previous morphologic studies show a continuity of the vitreous fibers with the basement membrane of the ciliary epithelium and the Mueller cells.32 The ultrastructural, biochemical, and biophysical properties of vitreous in situ, the nature of the vitreoretinal interface, and other aspects of vitreous physiology (growth, turnover, homeostasis, and its topology) are all points of great clinical importance deserving further investigation. The techniques and results outlined here may be helpful in such studies.

Key words: collagen, type I collagen, type V collagen, type IX collagen, vitreous biochemistry

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References

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