# Type VIII Collagen from Bovine Descemet's Membrane: Structural Characterization of a Triple-Helical Domain†

Rajesh Kapoor,‡,§ Paul Bornstein,§ and E. Helene Sage*,‡

*Departments of Biological Structure and Biochemistry, University of Washington, Seattle, Washington 98195*

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ABSTRACT: Bovine corneal Descemet's membrane (DM) was subjected to limited pepsin digestion. Soluble native collagens were fractionated by differential salt precipitation, and a mixture of type V collagen and collagenous fragments with a chain $M_r$ of 50 000 (50K) was obtained at a concentration of 1.5 M NaCl. Further purification of the 50K collagen by molecular sieve and high-performance liquid chromatography resulted in the isolation of two non-disulfide-bonded polypeptides, 50K-A and 50K-B, which were susceptible to several neutral proteases, including bacterial collagenase. By the criteria of peptide mapping, amino acid composition, and N-terminal sequence analysis, 50K-A and 50K-B were structurally dissimilar, although both chains contained Gly-X-Y repeats. 50K-A and 50K-B were immunologically and structurally distinct from collagen type I, III, IV, V, and VI. Immunohistochemical studies of bovine ocular tissue showed preferential distribution of the collagen containing the 50K fragment in the DM, with a more disperse arrangement of apparently interconnecting fibrils in the corneal stroma. Type VIII collagen isolated from the culture medium of metabolically radiolabeled bovine corneal endothelial (BCE) cells and its pepsin-resistant $M_r$ 50 000 domain(s) both cross-reacted with antisera to 50K polypeptides from the corneal DM. Additionally, the CNBr peptide maps of pepsin-resistant $M_r$ 50 000 polypeptides of type VIII collagen isolated from BCE cells and bovine corneal DM were highly similar. We therefore suggest that the 50K-A and 50K-B polypeptides isolated by limited pepsin digestion of bovine DM represent collagenous domains of type VIII collagen, a structurally unique member of the collagen family that is an integral component of specialized extracellular matrices produced by endothelial cells.

Basement membranes are extracellular matrices that form a physical barrier between different cell types or between cells and their underlying stroma. Descemet's membrane, a morphologically unique BM[1] in the cornea of the eye, is secreted in part by the corneal endothelial cells (Kefalides et al., 1976; Hay & Revel, 1969). Its thickness increases severalfold during development, and the final size of the DM varies from 3 μm in young humans (Murphy et al., 1984) to 17 μm in adult bovine corneas (Lee & Davison, 1984).

Ultrastructural and biochemical studies on BM of different extraocular tissues have revealed similarities in their major molecular components, including type IV collagen, laminin, and heparan sulfate proteoglycan, and in their general morphology (Carlson et al., 1981; Chung et al., 1976; Kefalides & Denduchis, 1969). In contrast, DM has been shown ultrastructurally to consist of a hexagonal lattice that was composed of 160-nm dumbbell-shaped structures. This matrix component in turn contained 2-nm fibrils that were formed in the presence of the inhibitor of cross-link formation β-APN (Sawada et al., 1984). The collagenous components of DM have not been rigorously characterized, although preliminary studies indicate the presence of only minor amounts of type IV collagen (Newsome et al., 1981; Fujikawa et al., 1984) and substantial amounts of a collagen suggested to be type VIII (Labermeier & Kenney, 1983). The presence of type VIII collagen in DM is supported by the finding that corneal endothelial cells in vitro synthesize type VIII collagen (Sage et al., 1981a; Benya, 1980).

Type VIII collagen was originally isolated from the culture medium of bovine aortic endothelial (BAE) cells (and hence termed "EC" or endothelial collagen) as three non-disulfide-bonded chains of $M_r$ 177 000 (EC1), 125 000 (EC2), and 100 000 (EC3) (Sage et al., 1980, 1983a). Although this collagen was unusually protease sensitive, it contained a pepsin-resistant domain(s) of $M_r$ 50 000 (per chain). However, a detailed analysis of type VIII collagen was lacking due to limiting amounts in the cell culture medium. We therefore investigated an alternative source, the bovine DM, for the isolation of type VIII collagen. This paper describes the isolation and characterization of $M_r$ 50 000 collagenous polypeptides from DM. We show by several criteria that these polypeptides are structurally distinct from the interstitial (types I–III) collagens, from basal lamina (type IV) collagen, and from type V and VI collagens. On the basis of structural and immunologic criteria, they appear to be derived from type VIII collagen.

## Materials and Methods

*Isolation of Bovine DM.* Adult bovine eyes were obtained from Pel Freeze Biologicals (Rogers, AK) and were stored on

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‡ Department of Biological Structure.

§ Department of Biochemistry.

[1] Abbreviations: BM, basement membrane; β-APN, β-aminopropionitrile fumarate; BAE, bovine aortic endothelial; BCE, bovine corneal endothelial; DEAE, diethylaminoethyl; DM, Descemet's membrane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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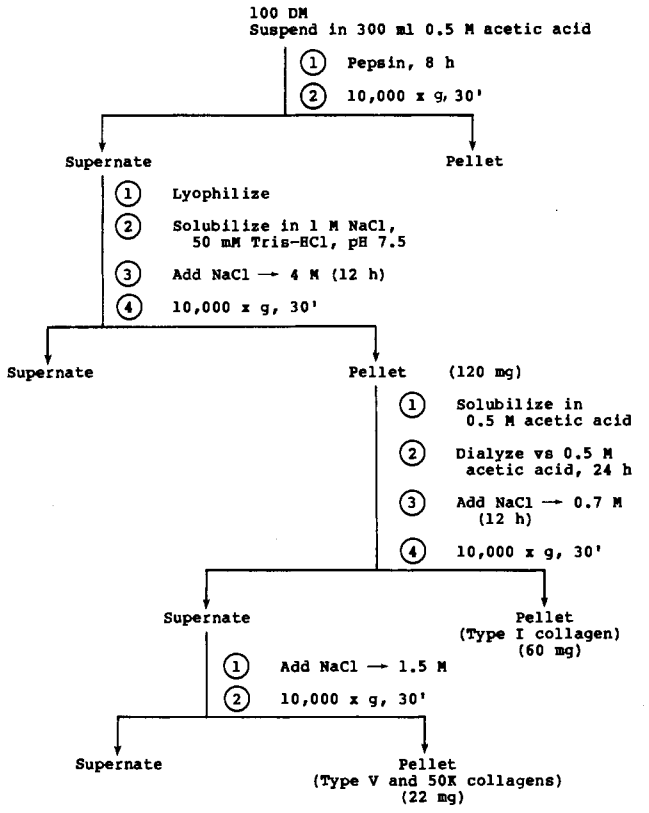


FIGURE 1: Procedure of isolation of 50K collagen from bovine Descemet's membrane. All procedures were performed at 4 °C.

dry ice. They were thawed in water containing the following protease inhibitors: 0.1 M caproic acid, 0.1 M EDTA, 5 mM *N*-ethylmaleimide, 5 mM benzamidine hydrochloride, and 1 mM $PhCH_2SO_2F$. The corneas were dissected and stored overnight at 4 °C in 10 mM Tris-HCl, pH 7.4, containing the above protease inhibitors. Subsequently, DM was separated from the corneal stroma with forceps, and cellular components associated with the DM were solubilized by detergent/DNase treatment as described by Carlson et al. (1978). The insoluble DM were then used as the starting material for the isolation of collagens.

*Isolation of Collagens from DM.* The isolation procedure (Figure 1) was carried out at 4 °C unless otherwise stated. Briefly, it consisted of dispersing the DM in 0.5 M acetic acid (tissue to acetic acid ratio of 1:4 w/v), followed by incubation with pepsin (0.5 mg/mL) (Sigma Chemical Co., St. Louis, MO) for 8 h. The extract was clarified by centrifugation at 10000*g* for 30 min, and the clarified supernatant was lyophilized. The lyophilized material was then resolubilized in 1 M NaCl and 50 mM Tris-HCl, pH 7.5. A small amount of insoluble material was removed by centrifugation at 10000*g* for 30 min. Solid NaCl was added with constant stirring to a final concentration of 4 M. This mixture was kept overnight at 4 °C, and the flocculant precipitate that had formed was collected by centrifugation at 10000*g* for 30 min. This precipitate was resolubilized in 0.5 M acetic acid and dialyzed against the same solution for 12 h, and the native collagens were isolated by fractional salt precipitation at 0.7 and 1.5 M NaCl (Miller & Rhodes, 1982). The collagens thus obtained were resolubilized in 0.5 M acetic acid, dialyzed against the same solution for 12 h, and lyophilized.

*Molecular Sieve Chromatography.* Further purification of the proteins that precipitated at 1.5 M NaCl was achieved by molecular sieve chromatography. Samples (2–3 mg) were dissolved in 1 mL of 1 M $CaCl_2$ and 50 mM Tris-HCl, pH 7.5, and denatured by heating at 55 °C for 20 min. This solution was clarified by centrifugation at room temperature for 2 min in a Beckman microfuge, followed by chromatography on Agarose A 1.5m (Bio-Rad, Richmond, CA). The column (1 × 90 cm) was eluted with 1 M $CaCl_2$ and 50 mM Tris-HCl, pH 7.5, at a flow rate of 12 mL/h. Approximately 1-mL fractions were collected and monitored by absorption at 230 nm.

*High-Performance Liquid Chromatography (HPLC).* Final purification of the fractions obtained by molecular sieve chromatography was performed by $C_{18}$ (Vydac, Separations Group, Hesperia, CA) reversed-phase HPLC as described by Qian and Glanville (1984) with the following modifications: the separation was carried out at 60 °C, with a linear gradient (20–40%) of acetonitrile in 0.1% trifluoroacetic acid. The flow rate of the column was 1 mL/min, and approximately 0.6-mL fractions were collected and monitored at 220 nm.

*Biosynthesis and Isolation of Type VIII Collagen.* Bovine corneal endothelial cells were provided by Dr. D. MacCallum (University of Michigan, Ann Arbor, MI) (MacCallum et al., 1982). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone, Sterile Systems, Inc., Logan, UT), supplemented with penicillin–streptomycin. Metabolic labeling of cells was carried out on nearly confluent cultures as previously described by Sage et al. (1980). The labeling medium contained no serum and was supplemented with 50 μg/mL sodium ascorbate, 64 μg/mL β-APN, and 25 μCi/mL L-[2,3-$^3$H]proline (35 Ci/mmol; New England Nuclear, Boston, MA). After a 20–24-h incubation with the isotope, the medium was removed into 5 mM *N*-ethylmaleimide, 0.2 mM $PhCH_2SO_2F$, and 2.5 mM EDTA. The medium was centrifuged at 5000*g* for 5 min to remove cellular debris, and solid ammonium sulfate was added with constant stirring at 4 °C to a final concentration of 50% (weight to initial volume ratio). Twelve hours later, the precipitate was collected by centrifugation at 20000*g* for 30 min at 4 °C. It was redissolved in a 6 M urea, 50 mM Tris-HCl, 0.2 mM $PhCH_2SO_2F$, and 2.5 mM EDTA buffer, pH 8.0, dialyzed against the same buffer at 4 °C (with at least three buffer changes), and chromatographed on DEAE-cellulose at 4 °C as described by Sage et al. (1983a). The unbound proteins from the DEAE-cellulose column were dialyzed against 0.5 M acetic acid at 4 °C. The dialyzate was divided into two equal parts: one was lyophilized, and the other was digested with pepsin (100 μg/mL) in a dialysis bag for 2 h at 4 °C followed by lyophilization.

*CNBr Cleavage.* Samples of 0.1–1 mg were dissolved in 1 mL of 70% formic acid containing 20 mg of CNBr. Nitrogen gas was bubbled through the solution for 30 s, and the tube was immediately sealed. The reaction mixture was incubated for 4 h at room temperature followed by molecular sieve chromatography on a PD10 column (1 × 10 cm) (Pharmacia Fine Chemicals, Piscataway, NJ). The column was eluted with 0.5 M acetic acid, and the void volume, which contained the major CNBr-digested products, was lyophilized and subsequently analyzed by SDS–PAGE.

*Two-Dimensional Peptide Mapping.* Samples were resolved on SDS–PAGE, and the gel was stained with Coomassie blue. The protein bands of interest were excised from the gel, washed extensively with propanol and methanol, and lyophilized. Each gel slice was then iodinated with the Bolton–Hunter reagent and digested with proteinase K, as described by Sage et al. (1981b). $^{125}$I-Labeled peptides were subjected to high-voltage electrophoresis in the first dimension followed by thin-layer chromatography in the second dimension.

*Amino Acid and N-Terminal Protein Sequence Analysis.* Samples (100 μg) were oxidized by performic acid as described by Hirs (1967), except that the reaction was terminated by rapid freezing and lyophilization. The products were subsequently hydrolyzed in 6 N HCl in vacuo at 110 °C for 24 h. The hydrolysate was lyophilized, and the analysis for amino acids was carried out on a Beckman Model 121C by Dr. W. R. Gray (University of Utah, Salt Lake City, UT). N-Terminal sequence analyses were carried out in a Beckman 890D spinning-cup sequencer with the automated procedure of Edman and Begg (1967), as described by Cruz et al. (1985).

*Antisera.* Antibodies against the 1.5 M NaCl precipitate (see Figure 1) were raised in female rabbits by subcutaneous and intramuscular injections, each containing 0.5 mg of protein in 0.5 mL of PBS mixed with an equal volume of complete Freund's adjuvant. Two further injections at 21-day intervals were given. Sera were collected 3 weeks after the final injection and tested by direct ELISA (Schuurs & Van Weeman, 1977) and by immunoblotting. Antisera were affinity absorbed against type V collagen isolated from DM. The protein (200 μg) was coupled to CNBr-activated Sepharose, and a 200-μL aliquot of antiserum was equilibrated with the column for 2 h at 4 °C. The unbound IgG was subsequently eluted with PBS.

Affinity-purified antibodies to type I, III, IV, V, and VI collagen were prepared as previously described by Sage et al. (1979, 1983b) and Trüeb and Bornstein (1984).

Immunoblotting was carried out according to the method of Towbin et al. (1979), as modified by Trüeb and Bornstein (1984), with the following changes: the proteins were transferred from the polyacrylamide gel to nitrocellulose at 4 °C for 2 h at 100 V. The washing procedures for nitrocellulose sheets were performed in 1% BSA/PBS, and the dried sheets were exposed to XRP-1 X-ray film (Kodak).

Tissue immunofluorescence on frozen corneal sections was performed with the assistance of Dr. T. Wight (University of Washington, Seattle, WA) as previously described by Wight et al. (1985), with the exception that all tissue sections were preincubated with 0.1 M acetic acid for 30–60 min prior to incubation with the antibody.

*Other Procedures.* SDS–PAGE was performed as described by Laemmli (1970) on slab gels containing 0.5 M urea, and proteins were visualized by staining with Coomassie Brilliant Blue R. Radiolabeled proteins were detected by fluorescence autoradiography as previously described by Sage and Bornstein (1982).

## Results

*Isolation of $M_r$ 50 000 Collagen from DM (50K DM).* A mild nonenzymatic procedure was used to separate DM from bovine corneas. Subsequent isolation of native collagens by short pepsin digestion (8 h at 4 °C) resulted in the solubilization of predominantly type I and V collagens and collagenous fragments of $M_r$ 50 000 (50K) chain molecular weight. Differential salt precipitation of this mixture under acid conditions yielded type I collagen in the 0.7 M NaCl fraction (Figure 2, lanes 1 and 3) and type V and 50K collagen in the 1.5 M NaCl fraction (Figure 2, lanes 2 and 4). Analysis of the 1.5 M NaCl fraction by SDS–PAGE showed that the mobility of the 50K component was unaltered in the presence of DTT (Figure 2, lanes 2 and 4) and that it was present in an approximately 1:1 ratio with type V collagen. The tissue remaining after pepsin digestion contained highly insoluble, disulfide-bonded components. Analysis of this residue by SDS–PAGE under reducing conditions revealed collagenous peptides ranging in molecular weight from 30 000 to 100 000.

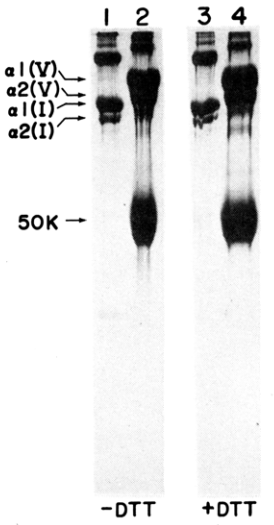


FIGURE 2: Analysis of the collagens obtained by differential salt precipitation of the pepsin extract of bovine DM. The salt fractionation was carried out in 0.5 M acetic acid, at 4 °C, and the fractions were analyzed by SDS–PAGE on an 8% gel. Proteins were visualized by staining with Coomassie blue. (Lanes 1 and 3) 0.7 M NaCl fraction; (lanes 2–4) 1.5 M NaCl fractions; (lanes 1 and 2) unreduced; (lanes 3 and 4) reduced.

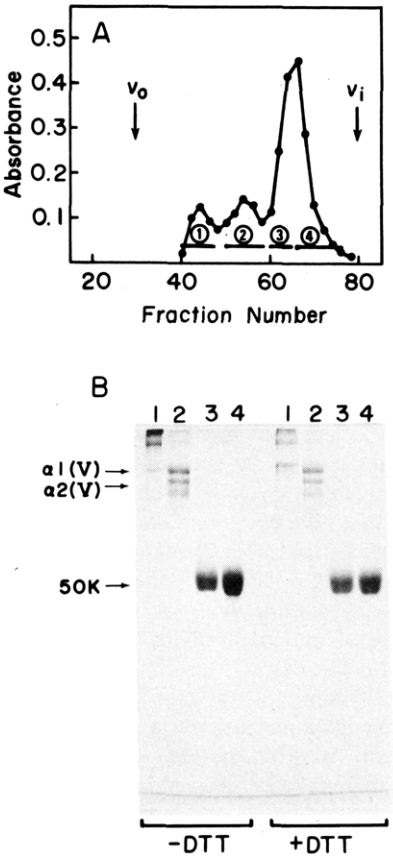


FIGURE 3: (A) Purification of the 1.5 M NaCl precipitate by chromatography on agarose 1.5m. Samples of 3–4 mg were dissolved in a 1 M $CaCl_2$ and 50 mM Tris-HCl buffer, pH 7.5, and denatured for 20 min at 55 °C prior to chromatography. The agarose column was equilibrated with the $CaCl_2$ buffer and eluted at a flow rate of 12 mL/h. Approximately 1-mL fractions were collected and monitored at 230 nm. Fractions were pooled as indicated by solid bars. (B) Lanes 1–4 correspond to the pooled fractions from the agarose column, after SDS–PAGE on an 8% gel in the absence and presence of DTT. The α chains of types I and V collagen are shown, and the 50K component has been identified.

It is likely that these peptides represent type IV and VI collagens, which were cross-linked in the tissue and required more

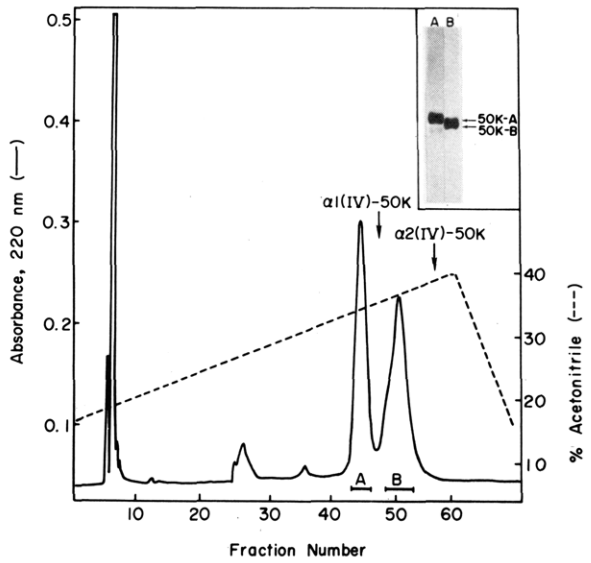


FIGURE 4: Resolution of the 50K component into two polypeptides, 50K-A and 50K-B, by HPLC. Samples of 200 μg were chromatographed on a Vydac C18 column at 60 °C with a linear gradient of acetonitrile (20–40%) in 0.1% trifluoroacetic acid. Approximately 0.6-mL fractions were collected and monitored by absorption at 220 nm. The solid bars indicate pooled fractions that were subsequently analyzed by SDS–PAGE on an 8% gel (inset). Peaks designated A and B correspond to 50K-A and 50K-B collagens. The elution positions of pepsin-treated bovine type IV collagen chains of $M_r$ 50 000 (α1-(IV)-50K, α2(IV)-50K) have been indicated.

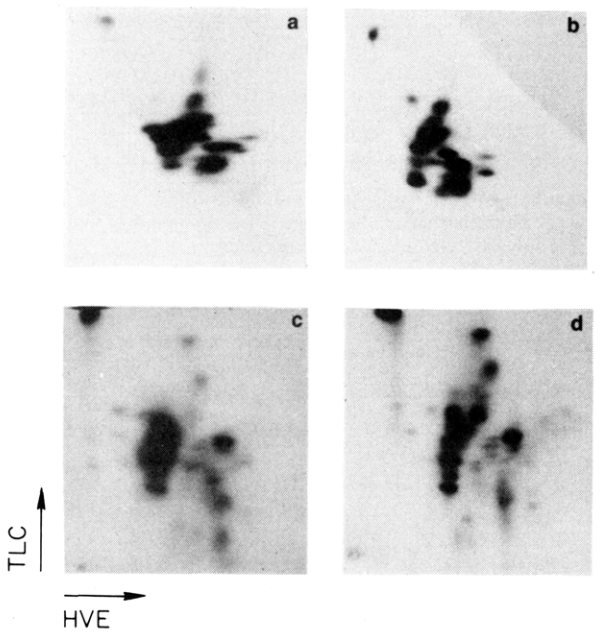


FIGURE 5: Comparison between $^{125}$I-labeled peptide maps of (a) 50K-A, (b) 50K-B, (c) α1(IV)-70K from human placenta, and (d) α1(IV)-50K from human placenta. The two-dimensional peptide mapping was performed following iodination and digestion with proteinase K, by the method of Sage et al. (1981b). HVE, high-voltage electrophoresis; TLC, thin-layer chromatography.

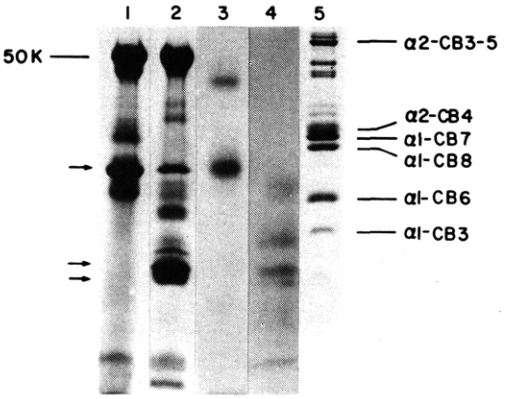


FIGURE 6: One-dimensional CNBr peptide mapping of 50K collagen from DM and pepsin-resistant $M_r$ 50 000 domain of type VIII collagen from BCE cell culture medium. Each sample was initially purified by HPLC prior to cleavage by CNBr. (Lane 1) 50K-A; (lane 2) 50K-B; (lane 3) [$^3$H]Pro-labeled $M_r$ 50 000 polypeptide (type VIII), HPLC peak A; (lane 4) [$^3$H]Pro-labeled $M_r$ 50 000 polypeptides (type VIII), HPLC peak B; (lane 5) type I collagen. Proteins were visualized after SDS–PAGE (12.5% gel in the presence of 50 mM DTT) by staining with Coomassie blue (lanes 1, 2, and 5) or fluorescence autoradiography (lanes 3 and 4). The CNBr-derived peptides of type I collagen have been identified.

severe conditions for extraction (data not shown).

Further purification of the 1.5 M NaCl fraction was achieved by molecular sieve chromatography, as shown in Figure 3. The first two peaks contained type V collagen. The leading edge of the third peak contained a mixture of 50K and a small amount of type V collagen, and the trailing edge consisted of pure 50K collagen (Figure 3B).

*Structural Studies.* Incubation with bacterial collagenase completely degraded the 50K polypeptides; in contrast, human skin collagenase did not degrade 50K under conditions where lathyritic rat skin type I collagen was cleaved specifically to A and B fragments (not shown). The 50K collagen was also partially degraded by mast cell protease, chymotrypsin, and trypsin (results not shown). The pattern of cleavage products obtained for 50K collagen after incubation with these enzymes was significantly different from that observed for collagen types I and IV (human placenta, $M_r$ 70 000 fragment) and thereby indicated that the 50K collagen was structurally distinct from these other collagen types.

HPLC of 50K collagen produced two peaks (Figure 4). Each peak contained a single component, 50K-A or 50K-B, as shown by SDS–PAGE (Figure 4, inset). Pepsin-derived fragments of the α1 and α2 chains of bovine type IV collagen ($M_r$ 50 000) were also resolved into two peaks by HPLC (arrows, Figure 4). Analysis of these peaks by SDS–PAGE showed that 50K-A and 50K-B did not coelute on HPLC with any peptide derived from type IV collagen (data not shown).

A two-dimensional peptide mapping technique was also used to characterize 50K-A and 50K-B. The resulting "fingerprints" of each polypeptide, after radioiodination and protease cleavage, showed differences between the two proteins and suggested that 50K-A was not derived from 50K-B (Figure 5a,b). Similar mapping of α1(IV)-50K and α1(IV)-70K peptides of human placenta type IV collagen revealed marked dissimilarities between these and the 50K-A and 50K-B polypeptides (Figure 5c,d, compare with panels a and b). The similarity between the peptide map of α1(IV)-50K and that of α1(IV)-70K supports the conclusion that the $M_r$ 50 000 peptide was derived from the $M_r$ 70 000 peptide of α1(IV) (MacWright et al., 1983).

The CNBr digestion products of 50K-A and 50K-B are shown in Figure 6. 50K-A (lane 1) contained a major CB peptide that migrated between α1-CB8 and α1-CB6 (single arrow). 50K-B (lane 2) contained two major CB peptides that migrated below α1-CB3 (double arrows). The pepsin-resistant $M_r$ 50 000 collagenous domain(s) of type VIII collagen, produced by BCE cells in vitro, was subjected to HPLC. The elution pattern resembled that shown for the DM 50K collagen (Figure 4). Peaks A and B were subsequently digested with CNBr, and the products were analyzed by SDS–PAGE, as shown in Figure 6 (lanes 3 and 4). Comparison of 50K-A

50K-A Pro-Met-Asp-Leu-X-Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Lys-Hyp-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly

50K-B Arg-Gly-Glu-Gln-Gly-Pro-Arg-Gly-Glu-Hyp-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-X-Hyp-Gly-Leu-Hyp-Gly-Gln

Gly-Ile-Hyp-Gly-Val-Ala-Gly-Cys-Hyp-Gly-Pro

FIGURE 7: N-Terminal sequence analysis of 50K-A and 50K-B. The purified polypeptides were subjected to automated Edman degradation, and the products were analyzed by HPLC, as described under Materials and Methods. X marks the residues that were not identified. Interruptions in the Gly-X-Y sequence are underlined.

Table I: Amino Acid Composition of $M_r$ 50 000 Collagen Chains Purified from Bovine Descemet's Membrane[a]

| | residues/1000 residues | | | residues/1000 residues | |
|---|---|---|---|---|---|
| | 50K-A[b] | 50K-A[b] | | 50K-A[b] | 50K-A[b] |
| Asx | 42.9 | 19.3 | Cys[d] | 5.9 | 2.5 |
| Thr | 23.2 | 9.4 | Ile | 18.9 | 32.1 |
| Ser | 21.5 | 9.2 | Leu | 72.0 | 67.6 |
| Glx | 83.3 | 85.1 | Tyr | 12.2 | 6.1 |
| Pro | 90.8 | 109.1 | Phe | 21.6 | 16.2 |
| Hyp | 117.2 | 152.3 | Hyl | 23.5 | 20.9 |
| Gly | 315.4 | 369.4 | Lys | 23.1 | 22.6 |
| Ala | 53.5 | 37.0 | His | 8.1 | 4.4 |
| Val | 32.0 | 31.9 | Arg | 35.8 | 19.1 |
| Met[c] | 7.6 | trace | Trp | ND[e] | ND |

[a] The analyses shown represent the average of two determinations. [b] 50K-A and 50K-B were purified by HPLC, as shown in Figure 4. [c] Determined as methionine sulfone. [d] Determined as cysteic acid. [e] ND, not determined.

(lane 1) with 50K(VIII)-A (lane 3) revealed a similar peptide pattern, including the major cleavage product (single arrow). 50K-B (lane 2) and 50K(VIII)-B (lane 4) were nearly equivalent in their distribution of cleavage products. The overall similarity of these one-dimensional peptide maps provided evidence that the 50K pepsin-resistant collagenous fragments from bovine Descemet's membrane were derived from type VIII collagen produced by bovine corneal endothelial cells.

*Amino Acid and Sequence Analysis.* The amino acid composition of 50K-A and 50K-B are shown in Table I. The levels of hydrophilic amino acids were generally higher, and the content of Gly was lower, in 50K-A as compared to 50K-B. The ratio of Pro to Hyp was 1.29:1 and 1.39:1 in 50K-A and 50K-B, respectively. The amino acid compositions of $\alpha$1-(IV)-50K (Schuppan et al., 1984) and $\alpha$2(IV)-50K (MacWright et al., 1983) were distinct from that of either 50K-A or 50K-B from DM.

N-Terminal sequence analysis of 50K-A and 50K-B was also consistent with a collagenous triple-helical structure (Figure 7). 50K-A contained a short nonhelical region followed by a continuous Gly-X-Y sequence. In 50K-B, the Gly-X-Y sequence was characterized by a short discontinuity (Figure 7). The differences observed between the amino acid composition and N-terminal sequence of 50K-A and 50K-B further support the conclusion that these peptides are distinct from each other.

*Characterization of Antiserum to 50K Collagen.* Antiserum was raised in rabbits against the 1.5 M NaCl precipitate (see Figure 1), which contained a mixture of native type V and 50K collagens. A positive reaction was obtained by ELISA for the 50K collagen, up to a dilution of 1:10 000 (Figure 8). There was no measurable activity toward type IV or V collagen. Further characterization of the antiserum was performed by immunoblotting as shown in Figure 9. A positive reaction with 50K and a high molecular weight polypeptide (arrow), which migrated between $\alpha$1(I) and $\alpha$2(I), was apparent (lane 1). The antiserum did not react with bovine collagen type IV

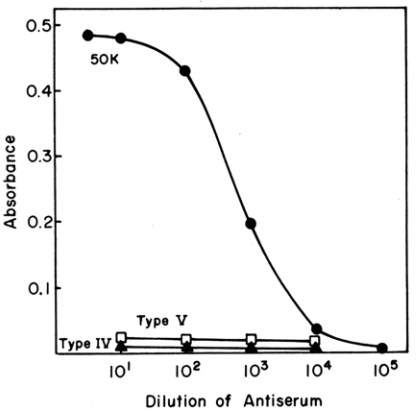


FIGURE 8: Characterization of antiserum to 50K-DM collagen isolated from bovine DM. Microtiter plates were coated with 5 $\mu$g of various collagens dissolved in 0.1 M acetic acid. Antiserum to the 50K collagen was serially diluted and reacted with the antigens on the microtiter plates.

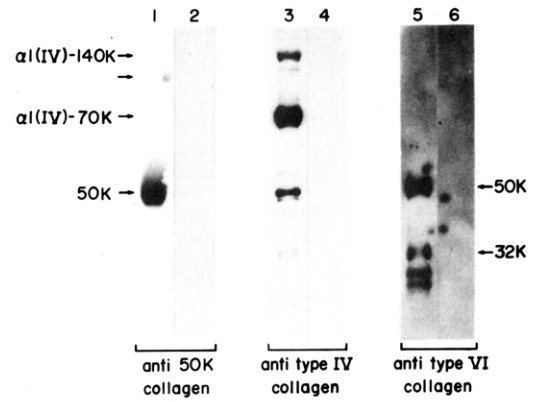


FIGURE 9: Immunoblots of pepsin-extracted collagens after exposure to various anti-collagen antibodies. Samples were resolved on 8% SDS–polyacrylamide gels and transferred to nitrocellulose as described under Materials and Methods. (Lane 1) 50K collagen; (lane 2) type IV collagen; (lane 3) type IV collagen; (lane 4) 50K collagen; (lane 5) type VI collagen; (lane 6) 50K collagen. Lanes 1 and 2 were exposed to anti-50K antibodies, lanes 3 and 4 were exposed to anti-type IV collagen antibodies, and lanes 5 and 6 were exposed to anti-type VI collagen antibodies. Fragments derived from pepsin digestion of types IV and VI, and the 50K collagen, have been identified. Unlabeled arrow indicates a fragment of approximate $M_r$ 95 000 that was recognized by the anti-50K collagen antiserum.

(lane 2) or with types I, III, V, and VI (data not shown). Conversely, antisera to collagen types IV and VI did not react with 50K or the peptide that migrated between $\alpha$1(I) and $\alpha$2(I) (lanes 4 and 6).

Type VIII collagen was isolated from the culture medium of BCE cells as three polypeptides [EC1 ($M_r$ 177 000), EC2 ($M_r$ 125 000), and EC3 ($M_r$ 100 000)] (Figure 10, lane 1). Pepsin digestion of this collagen produced $M_r$ 50 000 polypeptides (EC-50K, lane 2), as previously described by Sage et al. (1980, 1983a). Immunoblotting of these type VIII collagen chains showed a positive reaction of EC2 and EC-50K with anti-50K-antiserum (Figure 10, lanes 3 and 4). In addition, the $M_r$ of 50 000 collagenous polypeptide recovered from

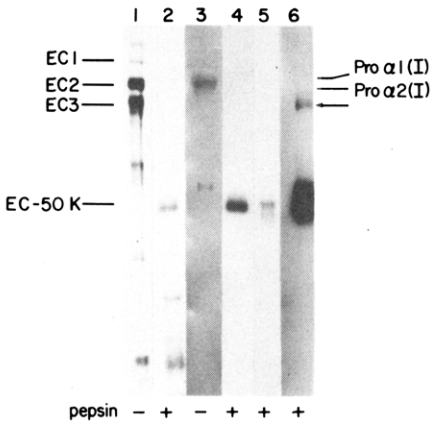


FIGURE 10: Immunological identity of 50K-DM collagen with type VIII collagen produced by BCE cells in vitro. Type VIII collagen was isolated from the culture medium of BCE cells as described under Materials and Methods. [$^3$H]Proline-labeled proteins that eluted unretarded from DEAE-cellulose were dialyzed against 0.5 M acetic acid and lyophilized. An aliquot of this material was, in addition, digested with pepsin at 4 °C for 2 h. Both fractions were analyzed by SDS–PAGE on an 8% gel in the presence of 50 mM DTT. Lanes 1 and 2 represent fluorescence autoradiograms of type VIII collagen before (lane 1) and after (lane 2) pepsin digestion. Type VIII collagen chains and the pepsin-resistant fragment(s) (EC-50K) have been identified. Lanes 3–6 represent an immunoblot of BCE cell derived type VIII collagen, after exposure to anti-50K DM collagen antiserum. (Lane 3) Sample as shown in lane 1; (lane 4) sample as shown in lane 2; (lane 5) pepsin digest of BCE cell layers; (lane 6) standard native 50K collagen from DM, used as immunogen for the anti-50K DM collagen antiserum. Mobilities of standard type I procollagen chains are shown. Unlabeled arrow indicates a fragment of approximate $M_r$ 95 000 that was recognized by the anti-50K collagen antiserum.

pepsin digests of BCE cell layers (Sage et al., 1981a) also reacted positively with the anti-50K collagen antiserum (lane 5). We have therefore shown that BCE cell derived type VIII collagen and the 50K collagen isolated from bovine DM are immunologically related. The cross-reactivity between 50K collagen and the $M_r$ 95 000 polypeptide (Figure 9, lane 1, arrow) suggests that this component might be a precursor form of the 50K collagen that was retained after incomplete pepsin cleavage of the parent molecule. Since EC3 did not react with the antiserum (lane 3), it would appear that this chain does not contain the EC-50K domain(s) that exhibited a positive reaction as shown in lane 4 (Figure 10).

*Immunofluorescence.* Frozen sections of adult bovine corneas were exposed to antibodies specific for several different collagen types (Figure 11). Staining of the DM was observed with anti-type IV collagen IgG (panel a). In contrast, anti-type VI collagen antibodies stained primarily the corneal stroma (panel b). Intense staining of the DM was observed with anti-50K collagen antibodies (panel c). Immunofluorescence of calf cornea produced a similar distribution of the 50K collagen, although quantitatively the staining in the DM was much less intense (results not shown).

## Discussion

We have described the isolation and characterization of $M_r$ 50 000 collagenous polypeptides (50K-A and 50K-B) from a basement membrane, the bovine Descemet's membrane, a secretory product of corneal endothelial cells. Only a short pepsin treatment of the DM was required to isolate these polypeptides in the form of a collagenous fragment, which represented 10–15% of the total collagens extracted from this tissue; a second pepsin digestion of DM did not result in further extraction of this fragment. The presence of collagen types I and V in our preparation was indicative of a small amount of stromal contamination (Tseng et al., 1982; Davison et al., 1979; Lee & Davison, 1984). However, the $M_r$ 50 000 polypeptides (50K-A and 50K-B) were purified from other collagens by differential salt precipitation, molecular sieve chromatography, and HPLC. Biochemical characterization of these polypeptides revealed that they were structurally distinct from each other by the criteria of two-dimensional $^{125}$I-labeled peptide mapping, CNBr peptide mapping, amino acid composition, and N-terminal sequence analysis.

Biochemical studies have shown that both type IV and VI collagens contain $M_r$ 50 000, triple-helical collagenous domains, which were released from the parent molecule after pepsin digestion and/or disulfide bond reduction. Since we isolated 50K polypeptides from DM by pepsin digestion, it was necessary to show, by several criteria, that 50K-A and 50K-B were distinct from other collagen types that have been shown to be present in cornea. (1) The two-dimensional peptide maps of the 50K-A and 50K-B polypeptides were significantly different from those of the $M_r$ 50 000 and 70 000 polypeptides of type IV collagen. (2) Tests by ELISA and immunoblotting showed that affinity-purified antisera against type IV and VI collagen did not cross-react with 50K polypeptides. Conversely, antisera raised against the 50K polypeptides did not cross-react with bovine type I, III, IV, V, and VI collagen. (3) The 50K (VIII) polypeptide exhibited distinctive CNBr cleavage patterns, amino acid compositions, and amino acid sequences.

Although several studies on ocular tissue have shown that type IV collagen is present in both Bowman's and Descemet's membrane (Konomi et al., 1984; Kefalides, 1971; Newsome et al., 1981), type IV collagen does not appear to be a major component of DM. Immunohistochemical studies on bovine corneas have shown that DM is only faintly stained with antibodies to type IV collagen (Fujikawa et al., 1984; Linsenmayer et al., 1983; Fitch et al., 1982). Furthermore, Linsenmayer et al. (1984) have shown that type IV collagen is absent from the DM of the developing chick cornea and forms only a minor component of the membrane in the mature tissue. In our immunofluorescence studies, we found that both calf and adult DM contained small amounts of type IV collagen, whereas antibodies to type VI collagen stained only the corneal stroma. This result is in agreement with the studies of von der Mark et al. (1984), who showed that type VI collagen was

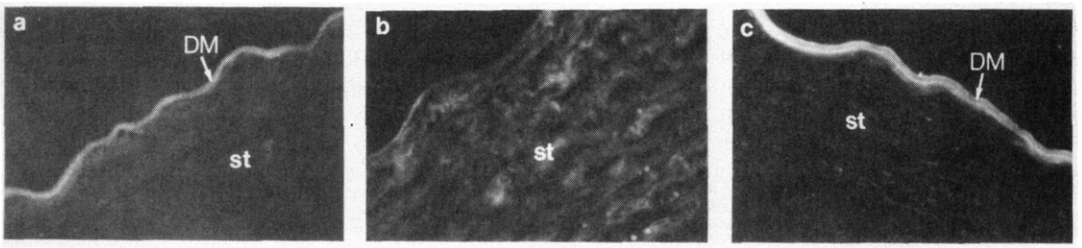


FIGURE 11: Immunofluorescence micrographs of bovine corneas showing Descemet's membrane (DM) and corneal stroma (st). All cryostat sections were incubated with 0.1 M acetic acid for 30–60 min at room temperature before exposure to antibody. Bovine cornea incubated with (a) anti-type IV collagen IgG, (b) anti-type VI collagen antibodies, and (c) anti-50K collagen antibodies. Original magnification 100×.

absent from basement membranes and was distributed largely as an interconnecting meshwork in connective tissue extracellular matrix. In contrast, an intense staining of the bovine DM was observed with anti-50K collagen antibodies. Since the intensity of staining of DM with this antibody was higher in adult tissue as compared with calf tissue, it appeared that the 50K collagen accumulated in DM with increasing age. This finding was supported by our observation that larger quantities of this collagen were extracted by pepsin digestion of adult bovine DM compared with fetal tissue.

Since in vitro studies have shown that corneal endothelial cells are the principal source of DM (Murphy et al., 1984), it was reasonable to assume that collagenous proteins of this membrane might be secreted by these cells. Most endothelial cells that have been studied have been shown to synthesize type VIII collagen (Sage et al., 1984). This collagen was originally described by Sage et al. (1980) from bovine aortic endothelial cells as three non-disulfide-bonded chains of $M_r$ 177 000–100 000. Pepsin digestion of type VIII collagen produced a collagenous fragment containing $M_r$ 50 000 polypeptides. In addition, Benya (1980) suggested that rabbit corneal endothelial cells synthesized type VIII (EC) collagen, although definitive peptide maps were not provided. In this study, we isolated type VIII collagen from bovine corneal endothelial (BCE) cells and showed that (1) antisera to the 50K polypeptides isolated from bovine DM cross-reacted with native type VIII collagen and its $M_r$ 50 000 pepsin-derived fragment and (2) CNBr peptide maps of 50K-A (DM), 50K-B (DM), and $M_r$ 50 000 polypeptides obtained by pepsin digestion of BCE cell type VIII collagen were very similar. In addition, the tissue-extracted fragment containing 50K-A and 50K-B polypeptides was nondisulfide-bonded, collagenase-sensitive, and pepsin-resistant and was localized to the DM by immunofluorescence. These data strongly suggest that the 50K polypeptides isolated from DM are derived from type VIII collagen.

Although the $M_r$ 50 000 pepsin-resistant domains of type VIII collagen were tentatively described by Sage et al. (1983a) as linked in series by noncollagenous peptides to form "cassette"-like chain structures, the possibility of other structural arrangement cannot be discounted. It is not certain that all "cassettes" are structurally the same, since we have described in detail two structurally different $M_r$ 50 000 polypeptides (50K-A and 50K-B) that were derived from a larger form of type VIII collagen. Collagenous domains of approximate $M_r$ 50 000 are also present in type IV, VI, IX, and X collagen (Sage et al., 1979; MacWright et al., 1983; Schuppan et al., 1980; Quarto et al., 1985; Schmid & Conrad, 1982; Gibson et al., 1982; Trüeb & Bornstein, 1984; van der Rest et al., 1985; Reese & Mayne, 1981; Reese et al., 1982). These collagenous domains are derived from collagen molecules representing a unique class of genes (Lozano et al., 1985) that are fundamentally different from those encoding the interstitial collagen types (Yamada et al., 1980; Ohkubo et al., 1980).

As the DM is a very thick BM that must remain transparent to light, its molecular architecture is of particular interest. Ultrastructurally, the DM has been shown to consist of a hexagonal lattice within a series of lamellar stacks (Gross, 1985). Each lamella is composed of non-collagenous "knobs" joined together by collagenous rods. An extracellular matrix of fine collagen fibers and non-collagenous molecules surrounds the hexagonal lattices (Sawada, 1982; Sawada et al., 1984). Desmosine and isodesmosine, two cross-links commonly found in elastin, have been described in insoluble fractions of DM that did not resemble elastin biochemically (Heathcote et al., 1982). The structure and function of such unusual noncollagenous macromolecules in DM are not known. However, we suggest that the architectural integrity of the DM is dependent upon interactions between collagen moelcules, including type VIII collagen, and other macromolecules of the extracellular matrix.

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## REFERENCES

Benya, P. D. (1980) *Renal Physiol. 3*, 30–35.
Carlson, E., Brendel, K., Hjelle, T. J., & Meezan, E. (1978) *J. Ultrastruct. Res. 62*, 26–53.
Carlson, E. C., Meezan, E., Brendel, K., & Kenney, M. C. (1981) *Anat. Rec. 200*, 421–436.
Chung, E., Rhodes, R. K., & Miller, E. J. (1976) *Biochem. Biophys. Res. Commun. 71*, 1167–1174.
Cruz, L. J., Gray, W. R., Olivera, B. M., Zeikus, R. D., Kerr, L., Yoshikami, D., & Moczydlowski, E. (1985) *J. Biol. Chem. 260*, 9280–9288.
Davison, P. F., Hong, B.-S., & Cannon, D. J. (1979) *Exp. Eye Res. 29*, 97–107.
Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.
Fitch, J. M., Gibney, E., Sanderson, R. D., Mayne, R., & Linsenmayer, T. F. (1982) *J. Cell. Biol. 95*, 641–647.
Fujikawa, L. S., Foster, C. S., Gipson, I. K., & Colvin, R. B. (1984) *J. Cell Biol. 98*, 128–138.
Gibson, G. J., Schor, S. L., & Grant, M. E. (1982) *J. Cell. Biol. 93*, 767–774.
Gross, J. (1985) *NATO Conference on Biology of Invertebrate and Lower Vertebrate Collagens*, Como, Italy, June 1986 (Bairati, A., & Garrone, R., Eds.) pp 1–28, Plenum, New York.
Hay, E. D., & Revel, J. P. (1969) *Fine Structure of the Developing Cornea*, Karger, Basel, Switzerland.
Heathcote, J. G., Eyre, D. R., & Gross, J. (1982) *Biochem. Biophys. Res. Commun. 108*, 1588–1594.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59–62.
Kefalides, N. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 226–234.
Kefalides, N. A., & Denduchis, B. (1969) *Biochemistry 8*, 4613–4621.
Kefalides, N. A., Cameron, J. D., Tomichek, E. A., & Yanoff, M. (1976) *J. Biol. Chem. 251*, 730–733.
Konomi, H., Hayashi, T., Nakayasu, K., & Arima, M. (1984) *Am. J. Pathol. 116*, 417–426.
Labermeier, U., & Kenney, M. C. (1983) *Biochem. Biophys. Res. Commun. 116*, 619–625.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, R. E., & Davison, P. F. (1984) *Exp. Eye Res. 39*, 639–652.
Linsenmayer, T. F., Fitch, J. M., Schmid, T. M., Zak, N. B., Gibney, E., Sanderson, R. D., & Mayne, R. (1983) *J. Cell Biol. 96*, 124–132.
Linsenmayer, T. F., Fitch, J. M., & Mayne, R. (1984) in *The*

*Role of the Extracellular Matrix in Development* (Trelstad, R. L., Ed.) pp 145–172, Liss, New York.
Lozano, G., Ninomiya, Y., Thompson, H., & Olsen, B. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4050–4054.
MacCallum, D. K., Lillie, J. H., Scaletta, L. J., Occhino, J. C., Frederick, W. G., & Ledbetter, S. R. (1982) *Exp. Cell Res. 139*, 1–13.
MacWright, R. S., Benson, V. A., Lovello, K. T., van der Rest, M., & Fietzek, P. P. (1983) *Biochemistry 22*, 4940–4948.
Miller, E. J., & Rhodes, R. K. (1982) *Methods Enzymol. 82*, 33–64.
Murphy, C., Alvarado, J., & Juster, R. (1984) *Invest. Ophthalmol. Visual Sci. 25*, 1402–1415.
Newsome, D. A., Foidart, J.-M., Hassell, J. R., Krachmer, J. H., Rodrigues, M. M., & Katz, S. I. (1981) *Invest. Ophthalmol. Visual Sci. 20*, 738–750.
Ohkubo, H., Vogeli, G., Mudryj, M., Avvedimento, V. E., Sullivan, M., Pastan, I., & de Crombrugghe, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7059–7063.
Qian, R.-G., & Glanville, R. W. (1984) *Biochem. J. 222*, 447–452.
Quarto, N., Cancedda, R., & Descalzi-Cancedda, F. (1985) *Eur. J. Biochem. 147*, 397–400.
Reese, C. A., & Mayne, R. (1981) *Biochemistry 20*, 5443–5448.
Reese, C. A., Wiedemann, H., Kühn, K., & Mayne, R. (1982) *Biochemistry 21*, 826–830.
Sage, H., & Bornstein, P. (1982) *Methods Enzymol. 82*, 96–127.
Sage, H., Crouch, E., & Bornstein, P. (1979) *Biochemistry 18*, 5433–5442.
Sage, H., Pritzl, P., & Bornstein, P. (1980) *Biochemistry 19*, 5747–5755.
Sage, H., Pritzl, P., & Bornstein, P. (1981a) *Arteriosclerosis (Dallas) 1*, 427–442.
Sage, H., Pritzl, P., & Bornstein, P. (1981b) *Collagen Res. 1*, 3–15.
Sage, H., Trüeb, B., & Bornstein, P. (1983a) *J. Biol. Chem. 258*, 13391–13401.
Sage, H., Farin, F. M., Striker, G. E., & Fisher, A. B. (1983b) *Biochemistry 22*, 2148–2155.
Sage H., Balian, G., Vogel, A. M., & Bornstein, P. (1984) *Lab. Invest. 50*, 219–231.
Sawada, H. (1982) *Cell Tissue Res. 226*, 241–255.
Sawada, H., Konomi, H., & Nagai, Y. (1984) *Eur. J. Cell Biol. 35*, 226–234.
Schmid, T. M., & Conrad, H. E. (1982) *J. Biol. Chem. 257*, 12451–12457.
Schuppan, D., Timpl, R., & Glanville, R. W. (1980) *FEBS Lett. 115*, 297–300.
Schuppan, D., Glanville, R. W., Timpl, R., Dixit, S. N., & Kang, A. H. (1984) *Biochem. J. 220*, 227–233.
Schuurs, A. H. W. M., & Van Weeman, B. K. (1977) *Clin. Chim. Acta 81*, 1–40.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Trüeb, B., & Bornstein, P. (1984) *J. Biol. Chem. 259*, 8597–8604.
Tseng, C. G. S., Smuckler, D., & Stern, R. (1982) *J. Biol. Chem. 257*, 2627–2633.
van der Rest, R., Mayne, R., Ninomiya, Y., Seidah, N. G., Chreitien, M., & Olsen, B. R. (1985) *J. Biol. Chem. 260*, 220–225.
von der Mark, H., Aumailley, M., Wick, G., Fleischmajer, R., & Timpl, R. (1984) *Eur. J. Biochem. 142*, 493–502.
Wight, T. N., Raugi, G. J., Mumby, S. M., & Bornstein, P. (1985) *J. Histochem. Cytochem. 33*, 295–302.
Yamada, Y., Avvedimento, E. V., Mudryj, M., Ohkubo, H., Vogeli, G., Irani, M., Pastan, I., & de Crombrugghe, B. (1980) *Cell (Cambridge, Mass.) 22*, 887–892.