Structure and biosynthesis of rabbit lens capsule collagen

Cholappadi V. Sundar-Raj and Ian L. Freeman*

The present study was designed to compare collagen synthesized by rabbit lens epithelial cells in culture with rabbit lens capsule collagen. Confluent monolayers of rabbit lens epithelial cells were established. Incorporation of [3H]-proline into glycoproteins secreted into the medium and cell surface components were analyzed in the presence of protease inhibitors. Gel filtration chromatography on sodium dodecyl sulfate-agarose (Bio-gel A-5m) of [3H]-labeled newly synthesized proteins by lens epithelial cells in culture resolved into a single precursor of approximate molecular weight of 160,000 daltons. Neither the medium nor the cell layer showed any evidence of low-molecular weight hydroxyproline-containing material. Limited pepsin digestion of this material cleaved the higher molecular weight chains into smaller components ranging from 25,000 to 110,000 daltons. Pepsin digestion and direct extraction of the collagenous components of the rabbit lens capsule revealed materials of high-molecular weight proteins similar to that synthesized in culture. Low-molecular weight (55,000 daltons) protein was only detected in lens capsules after prolonged pepsin digestion. S-Carboxylation of the lens capsules collagens did not affect their mobilities, but repepsinization gave rise to 110,000 dalton protein, although no significant changes in the amino acid composition were noticed. The absence of synthesis of low-molecular weight protein by cell culture and the presence of low-molecular weight components only after prolonged pepsin digestion of lens capsule could be the result of unusual susceptibility of the basement membrane collagens to pepsin attack. (Invest Ophthalmol Vis Sci 23:743-756, 1982.)

Key words: lens capsule, pepsin digestion, protein structure, epithelial cells, tissue culture, collagen biosynthesis

Recent studies on the structure and biosynthesis of basement membranes have led to the conclusion that the collagenous components of the basal laminae belong to a unique family of collagens.1 Kefalides2 and more recently Dehm and Kefalides3 used limited pepsin digestion to examine the internal structure of anterior lens capsule and reported the presence of a major membrane collagenous component slightly larger in size (110,000 daltons) than an interstitial collagen α-chain (95,000 daltons). Their work indicated that this component, α1(IV)-chain, originated from a molecule composed of three identical chains, which they have described by the molecular formula (α1(IV))3. Similar studies on kidney glomerular basement membrane and Descemet's membrane of the cornea indicated that the α-chains of type IV collagen were also prominent constituents of these basement laminae. Several biosynthetic studies5–7 have apparently corroborated this information and have led to the suggestion that
the collagenous constituents of basement membranes are synthesized as a homogeneous population of type IV molecules, analogues to the precursor molecules (procollagens) of the interstitial collagens. Unlike the interstitial collagens, however, the type IV procollagenous species is thought to be incorporated into the molecular structure of the basement membrane without "clipping" to α-sized chains. 

In contrast to these studies, other investigators, who have examined lens capsule and glomerular basement membrane by means of limited pepsin digestion, have found considerable heterogeneity in the denaturation products of the soluble collagens.

Even greater heterogeneity was found by investigators who examined the collagenous components of basement membranes without the use of proteases. Extraction of reduced and alkylated basement membranes in detergent-containing solvents produced collagen-like components with a high degree of heterogeneity, ranging in molecular size from 25,000 to 220,000 daltons.

Thus, in spite of extensive work over the past several years, characterization of the collagenous constituents of basement membranes is not yet complete, nor have the present studies resulted in a consensus concerning the nature and molecular organization of the components within the membrane.

To inquire into this controversy, we examined the collagenous components of anterior lens capsules from adult rabbits. We also examined the nature of the collagenous components synthesized by monolayer cultures derived from rabbit lens epithelial cells. We hoped that this combined biosynthetic and analytical approach would enable us to clarify the nature of material synthesized and would allow us to compare the synthetic products of the cultured cells with the material actually laid down in the basement membranes. We hoped also to identify those heterogeneities that were due to the methods currently used to study basement membrane collagen. Much of this work has been presented previously in preliminary form.

**Materials and methods**

**Isolation and purification of anterior lens capsule.** In general, each preparation started with 300 frozen mature rabbit eyes (Pel-Freez Biologicals, Inc., Rogers, Ark.). The cornea of each eye was removed. The iris was picked up with tweezers and pulled out at its roots. By squeezing the back of the eye, we extruded the vitreous body and lens onto a paper towel, where as much vitreous as possible was removed by dissection. The lens and the small amount of remaining attached vitreous was placed in a beaker of acetic acid in an ice bath. When all the lenses were dissected, they were transferred to a large Erlenmeyer flask, covered with acetone, and refrigerated at 4°C. The acetone was changed two or three times during the next 24 hr. The lenses became hard and white, and the lens capsule appeared as a transparent film surrounding the white lens protein and was readily removed. Only the central anterior lens capsule was collected. This usually contained small amounts of adherent lens protein, which appeared white. The anterior capsules were ultrasonicated alternately in distilled water and ice-cold acetone five times for 1 min in a Branson Model 220 ultrasonicator and were fragmented to small particles with two or three pulses of an ultra-Turrax homogenizer (Tekmar Co., Cincinnati, Ohio) prior to final washing in deionized water. The resultant transparent flakes were lyophilized. Previous studies indicated that this procedure removed a proportion of the noncollagenous components of lens capsule, but the collagenous component remained intact and the resultant lens capsule preparation was found to be exceedingly clean when examined both by microscopy and by chemical analysis.

**Pepsin-derived lens capsule collagens.** Lens capsule flakes were suspended in 0.5M acetic acid (500 ml/gm lens capsule), and sufficient pepsin (Worthington Biochemical Corp., Freehold, N.J.) was added to give an enzyme substrate ratio of 1:10. After shaking for 72 hr on a Burrel Wrist-Action Shaker at 4°C, the solubilized lens capsule collagens were purified according to the method described by Chung et al. Solid NaCl was added to 5%, and the collagenous materials that precipitated were further purified by resolubilization in phosphate buffer and reprecipitation with 20% ethanol in phosphate buffer, pH 7.4, a modification of the method of Church et al.

**Reduction and alkylation.** Lens flakes or purified collagens (approximately 60 mg) were resuspended in 1.0M Tris-HCl buffer, pH 8.6. Nitro-
Table I. Amino acid analysis of lens capsule and isolated collagen components in residues (1000 amino acid residues)*

<table>
<thead>
<tr>
<th>Amino Acid Residue</th>
<th>Whole lens capsule</th>
<th>Pepsin-derived collagen(s)</th>
<th>S-Carboxymethylated collagens re-pepsinized</th>
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</thead>
<tbody>
<tr>
<td>3-Hydroxyproline</td>
<td>4</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>4-Hydroxyproline</td>
<td>99</td>
<td>136</td>
<td>135</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>55</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td>Threonine</td>
<td>28</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Serine</td>
<td>43</td>
<td>31</td>
<td>31</td>
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<tr>
<td>Glutamic acid</td>
<td>87</td>
<td>79</td>
<td>77</td>
</tr>
<tr>
<td>Proline</td>
<td>67</td>
<td>68</td>
<td>64</td>
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<tr>
<td>Glycine</td>
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<td>315</td>
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</tr>
<tr>
<td>Alanine</td>
<td>46</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>Half cysteine</td>
<td>11</td>
<td>4</td>
<td>(4)</td>
</tr>
<tr>
<td>Valine</td>
<td>31</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Methionine</td>
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<tr>
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<tr>
<td>Tryptophan</td>
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<td>—</td>
</tr>
<tr>
<td>Arginine</td>
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</tr>
<tr>
<td>Total</td>
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<tr>
<td>% Glycosylation of OHLys residues</td>
<td></td>
<td>91</td>
<td>94</td>
</tr>
<tr>
<td>GGH/GH</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
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</table>

GGH = glucosylgalactosylhydroxylysine; GH = galactosylhydroxylysine.

*Note the high contents of hydroxyproline, hydroxylysine, and leucine, and the low content of alanine and the presence of small amounts of 3-hydroxyproline. The analyses indicate that lens capsule comprises about 60% collagenous material. No corrections were made in these analyses for hydrolytic losses. All hydroxylysine residues were glycosylated to greater than 90%, and most of this was GGH. The results presented here are averages of nine analyses—three for hydroxyproline, three for glycine, and three for the remaining amino acids. The analysis of purified lens capsule material is similar to that reported by other studies.4

gen was bubbled through the solution and mercaptoethanol was added to 5% (w/v). After further displacement with nitrogen, the tubes were sealed and shaken overnight. The tubes were opened and iodoacetic acid (400 mg) dissolved in 1M NaOH (1 ml) was added; nitrogen was bubbled through the solutions and the tubes were corked, wrapped in aluminum foil, and kept in the dark for 3 hr. The resultant materials were placed in a dialysis bag and dialyzed against 0.2M acetic acid for 48 hr prior to lyophilization.

Time course of pepsin attack on lens capsule. To examine the effects of pepsin on solubilization of the lens capsule collagenous components, lens capsule flakes (12 mg) were suspended in 0.5M acetic acid (6 ml) and 1.2 mg of pepsin (Worthington) were added and the mixture was placed on the shaker at 4°C. Aliquots of 600 μl in duplicate were removed at 3, 6, 16, and 25 hr and were then frozen and lyophilized for gels.

Analytical procedures. Amino acid and glycoside analysis were carried out according to the procedures described by Freeman.23 The chain composition of each collagenous fraction was evaluated by means of a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) tube system (Bio-Rad Laboratories, Richmond, Calif.) and the protease-free collagenase pretreatments described.27 In addition, 7.5% crosslinked tube gels were used to evaluate the effects of pepsin on lens capsule with respect to time. For biosynthetic studies, 5% to 15% gradient slab gels or 5% discontinuous gradient slab gels with 3% stacking gels were used in a Bio-Rad Model 220 vertical electrophoresis cell. Purified bovine type I collagen obtained from calf skin and the Bio-Rad low–molecular weight standards were used for internal standardization of the slab and tube gel procedures.

The molecular size of the rapidly labeled components released into the medium and those associated with the cells were examined by gel filtration.
Fig. 1. SDS-PAGE patterns (7.5% crosslinked) of whole lens capsule. Note the presence of four to five components of molecular weights 150,000 to 180,000 daltons (gel 1). In the absence of reducing agent no material entered the gel (gel 2). Bacterial collagenase destroyed all bands entering the gel (gel 3), and chymotrypsin gave rise to a single band of apparent molecular weight of 180,000 daltons (gel 4). No low-molecular weight material was found. Gel 17, Calf-skin collagen standard. The diffuse band in gel 3 was bacterial collagenase used in the test.

Examination of newly synthesized collagens. For each experiment, approximately forty 60 mm dishes containing confluent monolayers of lens epithelial cells were labeled with [3H]-proline (0.5 to 1.0 μCi/ml) in Dulbecco’s modified Eagle’s medium supplemented with ascorbic acid (50 μg/ml) to stimulate collagen synthesis and with β-aminopropionitrile (50 μg/ml) to inhibit collagen crosslinking.

In one series of experiments, after 4 hr of exposure to [3H]-proline, the medium was decanted, protease inhibitors were added (see below), and macromolecules were precipitated at 50% saturation ammonium sulfate. After resolubilization in SDS-agarose. Chromatography was performed at room temperature on a 95 by 1.5 cm diameter Bio-Gel A-5m column (6% crosslinked agarose). Descending elution was carried out at approximately 6.0 ml/hr with 0.2M Tris-HCl buffer, pH 7.4, containing 0.1% SDS and 0.3% sodium azide. Fractions of approximately 2 ml were collected. [3H]2O was added as an internal standard for each run, and the column was calibrated before and after experiments using the Bio-Rad column calibration kit. Fractions were assayed directly for total radioactivity and aliquots were removed for hydroxyproline assay. These aliquots (1.0 ml) were dialyzed exhaustively against water containing 5mM EDTA and 5 mM E aminoheptanoic acid prior to hydrolysis for 24 hr in 6M HCl at 105° C. The hydrolysates were evaporated to dryness and assayed for hydroxy-[3H]-proline by the method of Juva and Prockop.

Lens epithelial cell culture. Five albino rabbits weighing 2 to 3 kg were killed by intravenous injection of pentobarbital. After excision of the corneas, the anterior lens capsules were removed and placed in small (35 by 10 mm) tissue culture dishes containing Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, N. Y.) supplemented with fetal calf serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml). The capsules were unfolded and covered with sterile coverslips. When the initial growth of epithelial cells covered the entire surface of the dish (2 to 3 weeks), the cells were subcultured with Viokase (Gibco), as described by Sundar-Raj et al., into larger tissue culture dishes (60 by 15 mm). Confluent monolayers were achieved in 4 weeks from the initiation of the primary cultures. Cells were split 1:2 until fourth passage, when they were used for biosynthetic studies. Our cultures were grown at 37° C in a gas flow-humidified incubator in 5% CO2-95% air (Bellco Glass, Inc., Vineland, N. J.).
0.02M Tris-HCl, pH 7.4, containing 2% SDS, 0.1M urea, 50 mM dithiotreitol (DTT), materials were examined by chromatography on the A-5m SDS-agarose column described above.

Cell-associated materials were examined by collecting the cells with a rubber policeman in the Tris-urea-SDS-DTT buffer described above (2 ml/plate). After concentration of this extract with Aquacide (Calbiochem-Behring Corp., San Diego, Calif.) to approximately 10 ml, the concentrate was applied to the SDS-agarose column and analyzed as described above.

For detailed examination of collagen biosynthesis, labeling was continued for 48 hr. Harvested medium was brought to 50% saturation with respect to ammonium sulfate in the presence of protease inhibitors in order to precipitate all of the collagen-related proteins. The protease inhibitors used were N-ethylmaleimide (10 mM), phenylmethylsulfonyl fluoride (2 mM), and EDTA (2 mM). After resolubilization of the ammonium sulfate precipitate in phosphate buffer (pH 7.4, ionic strength 0.4), the collagenous materials were collected by reprecipitation with 20% (v/v) ethanol in fresh phosphate buffer, pH 7.4, prior to dialysis against water and lyophilization. This material was analyzed by SDS-PAGE, as described above. Limited pepsin digestion of some of these samples was carried out according to the method of Bellamy and Bornstein, and the presence of collagenous components was confirmed by digestion of samples with protease-free bacterial collagenase according to the method of Peterkofsky and Diegelmann. Slab gels were processed for fluorography according to the procedure of Bonner and Laskey and visualized by means of preflashed X-Omat film (Kodak, Inc., Rochester, N. Y.).

Results

Lens capsule preparation. It has previously been shown that the preparative method described in this article yielded extremely pure lens capsule material. Amino acid analysis of the preparation used in these studies (Table I) showed that the lens capsule flakes satisfied our previous criterion of purity and lacked contamination with lens capsule or vitreous proteins.

Chain composition of whole lens capsule. In the absence of reducing agent, none of the material of the rabbit lens capsule preparation entered the gel (Fig. 1, gel 2). In the presence of reducing agents, much of the material en-

Fig. 2. Components of lens capsule and preparation of collagen proteins. This photograph shows untreated lens capsule (gel 1), salt-precipitated purified lens capsule collagen (gel 5), and pepsin-prepared collagens (gel 5). S-Carboxymethylation of the collagens did not affect their mobilities (gel 9), but repepsinization (gel 13) gave rise to a major component at 110,000 daltons and destroyed the 80,000 dalton component. All the gels were run in the presence of the reducing agent, DTT.
Fig. 3. Effect of pepsin on lens capsule under limited digestion conditions. Even as early as 3 hr, a small amount of 55,000 dalton component was seen. As the digestion proceeded, this component increased and many other intermediate components were found. All the peaks were collagenase sensitive. The peak at 35,000 daltons in digestions at 3 to 25 hr is pepsin. This was removed by salt precipitation from the material electrophoresed at 72 hr.

tering the gel was concentrated near the top of the gel, indicating very high–molecular weight materials (Fig. 1, gel 1). However, four or five component chains of apparent molecular weight 120,000 to 180,000 daltons could readily be seen (Fig. 1, gel 1). No low–molecular weight protein chains (100,000 daltons) were evident. The collagenous nature of these chains was confirmed by a treatment with protease-free collagenase used in the test. Although pepsin digestion was found to produce a multiplicity of chain sizes (Fig. 3), chymotrypsin treatment gave rise to a single band of apparent molecular weight 180,000 daltons (Fig. 1, gel 4). Proteolytic attack on lens basement membranes have also been detailed by Uitto et al.13 and Mainardi et al.29

Pepsin-prepared lens capsule collagens.
The components of whole lens capsule and the effectiveness of pepsin digestion to release collagens was examined. The results of our preliminary experiments are shown in Fig. 2. The salt-purified lens capsule collagen (Fig. 2, gel 5) showed a major component at 160,000 daltons with several other low–molecular weight components at 80,000, 70,000 and 55,000 daltons. This is in marked contrast to the absence of low–molecular weight material of the untreated lens capsule (Fig. 2, gel 1). S-Carboxymethylation of these materials did not affect their mobilities (Fig. 2, gel 9), but re-pepsinization (Fig. 2, gel 13) gave rise to a major component at 110,000 daltons and destroyed the 80,000 dalton component. This will be discussed further (see Fig. 5).

To further examine the action of pepsin on the lens capsule preparation, we carried out the time-course experiment described. Fig. 3 shows the effects of the limited pepsin digestion on the lens capsule in this experiment. Even as early as 3 hr a small amount of low–molecular weight material (55,000 daltons) was seen, indicating rapid cleavage of the high–molecular weight chains (previously noted in Fig. 1, gel 1). As the digestion proceeded, the relative amount of this 55,000 dalton component was found to increase; in addition, many other intermediate components were found.

Limited pepsin digestion of whole lens capsule of 72 hr gave rise to some very high–molecular weight material that did not enter the gel and several major component chains of
approximately 160,000, 80,000, 70,000, and 50,000 daltons (Fig. 4, gel 5). The 160,000 and 80,000 dalton components were disulfide linked and were seen to be derived from components of molecular weight greater than 200,000 daltons in the absence of reducing agents (Fig. 4, gel 6). The collagenous nature of these components was confirmed by their destruction with protease-free bacterial collagenase (Fig. 4, gel 7) but not by chymotrypsin (Fig. 4, gel 8).

S-Carboxylation and repepsinisation. Reduction and alkylation confirmed the disulfide-linked nature of some of the components released from the lens capsule by a limited digestion with pepsin. The amino acid analysis of this material is shown in Table I. Fig. 5, gel 9, was run in the presence of a reducing agent, DTT; Fig. 5, gel 10, was run in its absence. These are substantially identical. Again, all components entering the gel were found to be susceptible to protease-free collagenase attack (Fig. 5, gel 11), and some of the collagenous components were sensitive to chymotrypsin treatment (Fig. 5, gel 12).

When the purified collagenous material was repepsinized under the same conditions as those used for the release of the collagenous components from the whole lens capsule, a major component at 100,000 daltons was seen (Fig. 6, gel 13). However, this material also showed higher molecular weight components running in the collagen \( \beta- \) and \( \gamma- \) chain positions (approximately 200,000 and 300,000 daltons) and lower molecular weight species at 70,000 and 55,000 daltons, and therefore appeared heterogeneous in nature (Fig. 6, gel 13). All these components were sensitive to protease-free collagenase (Fig. 6, gel 15) and were resistant to chymotrypsin attack.

Culture of rabbit lens epithelial cells. Fig. 7 is a phase-contrast micrograph of rabbit lens epithelial cell growth on a tissue culture dish after the anterior lens capsule had been placed over the dish. All the cells had typical polygonal shaped epithelial structure and were contact inhibited as they formed monolayer culture. Unlike fibroblasts, lens epithelial cells in subculture required longer incubation with pancreatin in order to lift them up from the dish. These cells did not exhibit any additional
Fig. 5. S-Carboxymethylated lens capsule collagens. Reduction and alkylation confirmed the disulfide-linked nature of some of the components described in Fig. 3. Gel 9 was run in the presence of reducing agent DTT; gel 10 was run in its absence. These are substantially identical. Gel 11, Collagenase-treated sample; gel 12, chymotrypsin-treated sample; gel 17, calf-skin collagen standard. The band in gel 11 was bacterial collagenase.

Fig. 6. Repepsinization of lens capsule collagens. Repepsinization gave rise to a major component at 110,000, similar to that described by other investigators. However, this material also contained higher molecular weight species (300,000, 180,000, and 160,000 dalton components) and lower molecular weight species (70,000 and 50,000 daltons) and thus were heterogeneous in nature (gel 13). All components are collagenous (gel 15) and resistant to chymotrypsin attack (gel 16).
requirement for growth factors other than fetal calf serum present in the growth medium.

Gel filtration chromatography on SDS-agarose of [3H]-labeled proteins synthesized by lens epithelial cells in culture. SDS in the presence of reducing agents was used to solubilize the newly synthesized molecules produced during a 4 hr incubation with radiolabel. This method was included to investigate the possible conversion of the collagenous polypeptides into lower molecular weight species.

Examination of newly synthesized materials released into the medium and associated with the cells by SDS-agarose chromatography (Fig. 8) revealed that the hydroxy-[3H]-proline peaks were distinct from the remainder of the labeled polypeptides. Presumably, the presence of significant amounts of lower molecular weight material indicates the synthetic capacity of epithelial cells for proteins other than basement membrane collagens. These peaks may represent crystallins but were not investigated further.

It should be noted, however, that only about 6% of the total radioactivity was accounted for by hydroxyproline, indicating that collagenous polypeptides represent only a small, albeit important, fraction of the synthetic output of lens epithelial cells in culture.

Neither medium nor cell layer fractions showed any evidence of significant amounts of low-molecular weight hydroxyproline-containing material. The single hydroxyproline-containing peak of the material released into the medium was found to run on the column with an apparent molecular weight corresponding to about 180,000 daltons, whereas material extracted from association with the cells showed a high-molecular weight shoulder running to the left of the major
Fig. 8. Gel filtration chromatograph on SDS-agarose of \(^{3}H\)-labeled proteins synthesized by lens epithelial cells in culture. A, Medium proteins precipitated with 50% saturated ammonium sulfate. B, Cell-associated protein extracted as described in the text. Elution was carried out at approximately 6 ml/hr with 0.2M Tris-HCl, pH 7.4, containing 0.1% SDS and 0.3% NaN\(_3\). The column elution position of \(^{3}H\)_2O is indicated by \(V_t\). For medium: \(V_0\), fraction 22; calf-skin collagen standard \(\alpha\)-chain, 51-52; \(\beta\)-chain 39-40. Solid line, \(^{3}H\)-proline; dashed line, \(^{3}H\)-hydroxyproline.

This indicated the presence of hydroxy-\(^{3}H\)-proline-containing molecules larger than 180,000 daltons.

**SDS-PAGE of newly synthesized collagens by lens epithelial cells in culture.** The collaginous components released into the medium by monolayer cultures of lens epithelial cells were purified by ethanol precipitation and resolved on 5% to 15% gradient slab gels. They resolved into two major components running...
Fig. 9. Fluorograph of ethanol-purified medium collagens from lens epithelial cells in culture (SDS-gradient polyacrylamide gel, 5% to 15% crosslinked). A, Purified material; C, same material after limited-pepsin digestion; E, same material after digestion with protease-free collagenase. These samples were run in the presence of the reducing agent DTT. B, D and F, Respective samples run in the absence of reducing agents. Note (1) the disulfide-linked nature of some of the components both before and after pepsin treatment, (2) the generation of low-molecular weight chains by pepsin, and (3) the collagenous nature of all materials as shown by the absence of chains in samples E and F.

with apparent molecular weights of 120,000 to 140,000 daltons and two minor components, one running just slower than the two major chains and one running at about 180,000 daltons (see Fig. 9, well A). Running the gel in the absence of reducing agents showed the disulfide-linked nature of some of the newly synthesized materials (Fig. 9, well B). No lower molecular weight material (<100,000 daltons) was seen in these preparations, although we have seen such components in experiments where protease inhibitors were not included during purification of the lens cell collagens (unpublished data).

Limited pepsin digestion of this material cleaved the high-molecular weight chains into smaller components ranging in size between 25,000 and 110,000 daltons. Major components appeared at 80,000 and 50,000 daltons (Fig. 9, well C). Reproduction of this degradation profile was difficult, since in this study, the process was very sensitive to minor differences in enzyme concentration, time of reaction, temperature, etc. However, components of molecular size 110,000, 70,000, 55,000, and 25,000 daltons appeared to be constant degradation intermediates. Even after limited pepsin digestion, some high-molecular weight disulfide-linked components were seen (Fig. 9, well D). The collagenous nature of all these chains was confirmed by their susceptibility to protease-free collagenase (Fig. 9, wells E and F).

Further resolution of the newly synthesized components was achieved on a 5% slab gel (Fig. 10). The two bands seen in the previous figure resolved into four; thus a total of six bands were seen, ranging in molecular size from approximately 100,000 to 170,000 daltons (Fig. 10, well A). The disulfide-linked nature of the 160,000 dalton component was clearly seen, since a component appeared in the 300,000 dalton region in the absence of reducing agent (Fig. 10, well B). The heterogeneity produced by limited pepsin digestion was also readily apparent by the presence of multiple bands (Fig. 10, wells C and D). In this experiment, the 55,000 dalton component was a major product of limited pepsin cleavage. This component was found to run at the bottom of this 5% gel (Fig. 10, wells C and
D). Again, the protease-free collagenase destroyed all the component chains, thus confirming their collagenous nature (Fig. 10, wells E and F).

Discussion

A major difficulty associated with most studies of basement membrane collagens has been the extreme insolubility of these proteins, presumably because of the presence of disulfide-bonded aggregates composed of both collagenous and non-collagenous sequences. One approach to obtaining information about basement membranes such as the lens capsule has been limited pepsin digestion in attempts to isolate and characterize only triple-helical collagenous sequences. Although up to 70% of the total hydroxyproline of the membranes is released by appropriate digestion conditions, much of the controversy about the primary structure of basement membrane collagens has resulted from various interpretations of data derived by this method. Although pepsin treatment has been most useful in structural studies of the interstitial collagens, the work reported here leads to a conclusion similar to that of other authors, i.e., that basement membrane collagen contains several centrally located pepsin-sensitive sites. Whether these sites are composed of non-triple-helical sequences, which interrupt the triple helix, or whether the pepsin sensitivity results from local instability within the helix itself has not been demonstrated. The preparation method reported in our work demonstrated a series of collagen chains in the initial preparation and the generation of low-molecular weight fragments only on exposure to proteolytic attack. Because the pepsin-sensitive region could be generally susceptible to proteolytic cleavage, it is possible that a similar pattern of fragments could be generated by extraction procedures not involving protease inhibitors.

It is also conceivable that these same sites could be susceptible to cleavage by proteases in vivo, e.g., when inflammatory cells are in transit through basement membrane. This chain fragility could explain some of the confusion generated by the early work on membrane collagens. Indeed, degeneration of basement membranes by neutral proteases released from human leukocytes has recently been demonstrated by Uitto et al. and Mainardi et al. Although the work reported here shows the generation of similar-sized pepsin-derived molecular species to those of other investigators, the exact molecular size of the fragments is not clear because of the differences in electrophoretic systems used in the different studies. Detailed amino acid analysis of the individual components of lens capsule material has led Dixit and Kang to suggest the presence of two genetically distinct collagenous components with different pepsin susceptibilities in basement membranes. Although we did not investigate this possibility further in our isolated lens capsule preparation, we did find additional evidence for the presence of two genetically distinct chains by means of the biosynthetic system (see below). As pointed out by Sage et al. and Heathcote et al., resolution of the confusion with regard to the number of collagenous components originally incorporated into lens capsule can be obtained by study of the biosynthesis of the lens capsule collagen chains. The studies reported here indicate that interpretation of the biosynthetic data depends on the analytical system used to investigate the biosynthetic pathway. For example, the result of SDS-agarose column chromatography demonstrated the apparent synthesis of a single precursor of molecular weight of approximately 160,000 daltons, which was released into the medium, and a similar-sized collagenous component was found in association with the cell surface, although in this instance, some crosslinked higher molecular weight material was also found to be present. No lower molecular weight material was seen when the experiments were carried out in the presence of protease inhibitors. However, when the increased resolving of SDS slab gel electrophoresis was used, two closely spaced chains of molecular weight of approximately 150,000 were seen on 5% to 15% gradient gel. Similar doublets have been reported by several authors, using different basement membrane synthesizing systems.

However, the presence of minor collage-
nous components on the slab gels and the susceptibility of some but not all of these components to reducing agents suggested further attempts to resolve the multichain complex. We therefore reran samples on a 5% cross-linked slab gel. Under these conditions, four separate components were resolved. Some of these components were disulfide linked. In common with a recent biosynthetic study of mouse tumor and parietal yolk sac tissue, our interpretation of the presence of these chains is that we are showing a mixture of type IV procollagens and type IV collagens. It should be noted that the type IV collagens are larger in size than the chains of the fibrous collagens and that these collagens can be treated by proteolytic enzymes and brought down to the approximate size of interstitial chains. The simplest explanation for these data is that the larger peptides are precursors for the smaller chains and that the conversion of the larger chains to the smaller ones is inefficient in vitro. Pepsin digestion studies indicated that the lower molecular weight chain (120,000 daltons) was degraded more extensively by the enzyme than the 140,000 dalton chain. The larger chains appeared to be converted to a slightly smaller component migrating in position between these two chains. The more extensive degradation of the 120,000 dalton chain could be due to cleavages in the helical region of the molecule or to cleavage of more extensive nonhelical sequences within this chain. Our biosynthetic system yielded identical results to those reported for the mouse tumor and parietal yolk sac systems. This difference in pepsin susceptibility can be interpreted as an indication of differences in the primary structures of the two newly synthesized chains. This would mean that they were different gene products. In the biosynthetic studies of the basement membrane collagens synthesized by mouse tumor and parietal yolk sac, the two newly synthesized collagen chains were designated as pro α1 type IV and pro α2 type IV chains. However, in this work and in ours, further studies such as peptide mapping of cyanogen bromide or V-8 protease degradation products, immunologic studies, and perhaps amino acid sequence studies, are needed to confirm the genetically distinct nature of these two chains. These studies are now in progress.

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REFERENCES