Studies on the Assembly of the Rat Lens Capsule

BIOSYNTHESIS AND PARTIAL CHARACTERIZATION OF THE COLLAGENOUS COMPONENTS

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1. Isolated rat lens capsules synthesized hydroxy[³H]proline-containing polypeptides when incubated with [³H]proline. 2. The collagenous polypeptides synthesized during a 2 h incubation were analysed by both gel-filtration chromatography and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and shown to have an apparent mol.wt. of approx. 180000. 3. No evidence was obtained for conversion of these polypeptides into a lower-molecular-weight species in experiments where capsules were labelled for 2 h and chased with non-radioactive proline for up to 22 h. However, a time-dependent incorporation of the 180000-mol.wt. species into a larger collagenous component was observed and this could be prevented by the inclusion of β -aminopropionitrile in the incubation medium. 4. The radioactive components synthesized by the capsules correspond to subunits of the intact lens capsule and the direct incorporation of the polypeptide of mol.wt. 180000 into deoxycholate-insoluble basement membrane was demonstrated.

Basement membranes are specialized connective-tissue structures that characteristically separate parenchymal cells from neighbouring interstitial connective tissue (Vracko, 1974). The relatively high carbohydrate content of these acellular hyaline sheets is indicated by their reaction with the periodic acid/Schiff reagent (Ashton, 1974). That these structures also contain a form of collagen was first established by the chemical and X-ray-diffraction analyses of ox lens capsule undertaken by Pirie (1951) and has since been confirmed by many workers (Spiro, 1967; Fukushi & Spiro, 1969; Kefalides, 1973; Daniels & Chu, 1975; Burgeson *et al.*, 1976; Dixit, 1978).

The precise form of the collagenous component is still unclear. Kefalides (1971) has isolated a discrete collagenous molecule from human glomerular basement membrane, sheep anterior lens capsule and sheep Descemet's membrane and this has been termed type-IV collagen (Kefalides, 1973) to distinguish it from types I, II and III, which are found in interstitial connective tissues (for review see Miller, 1976). Other workers have isolated collagens differing in composition and size from type IV (Trelstad & Kang, 1974; Daniels & Chu, 1975; Burgeson *et al.*, 1976). A number of glycoprotein components have also been extracted from basement membranes (Kefalides, 1973; Ohno *et al.*, 1975;

Johnson & Warfel, 1976) and some of these may be covalently linked to the collagen. Several workers (Hudson & Spiro, 1972*b*; Sato & Spiro, 1976; Freytag *et al.*, 1976; Taylor & Price, 1977) have demonstrated that treatment of reduced and alkylated glomerular basement membrane with detergents will solubilize a large number of polypeptides of different size and composition. On the basis of these observations, Spiro (1976) has put forward a model of basement-membrane structure in which cross-linked polypeptides of different length and containing differing proportions of collagen-like segments are layered over each other. At present, however, it is not possible to describe the molecular organization of the basement-membrane matrix with any certainty.

Biosynthetic studies offer a means of investigating basement-membrane components before they become fully integrated into the insoluble extracellular matrix. To date, a number of tissues have been used, including chick lens cells (Grant *et al.*, 1972*a,b*), isolated rat renal glomeruli (Grant *et al.*, 1975) and rat parietal yolk-sac endoderm (Clark *et al.*, 1975). These studies have indicated that a collagenous molecule analogous to the procollagens of interstitial connective tissues is the first-formed biosynthetic product. The results presented here suggest that the isolated rat lens capsule is a suitable system for investigating the biosynthesis of this component and its subsequent fate. A preliminary report of this work has been published (Heathcote *et al.*, 1977).

Abbreviations used: Tricine, *N*-[(2-hydroxy-1,1-bis-(hydroxymethyl)ethyl)glycine]; SDS, sodium dodecyl sulphate.

Experimental

Materials

Male Sprague-Dawley rats (130–160g body wt.) were supplied by the animal unit of the University of Manchester Medical School. Dulbecco's modification of Eagle's medium, foetal bovine serum, penicillin G, streptomycin and gentamycin were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. L-[5-³H]Proline (29Ci/mmol), L-[U-¹⁴C]proline (275mCi/mmol) and ³H₂O (5Ci/ml) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Acrylamide, *NN'*-methylenebisacrylamide and Bio-Gel A-5m were obtained from Bio-Rad Laboratories, Watford, Herts., U.K. Blue Dextran 2000 was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Tricine buffer, NCS tissue solubilizer (Amersham/Searle) and sodium deoxycholate were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Collagenase (grade 1) was purchased from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K., deoxyribonuclease 1 from Sigma (London) Chemical Co., London S.W.6, U.K., and $\alpha\alpha'$ -bipyridine from Koch-Light Laboratories, Colnbrook, Bucks., U.K. *N*-Ethylmaleimide, phenylmethanesulphonyl fluoride, cycloheximide, β -aminopropionitrile fumarate and dithiothreitol were bought from Sigma (London) Chemical Co. SDS (specially purified) was obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. and BDH Chemicals, Poole, Dorset, U.K. Embryonic-chick tendon and cartilage procollagens, labelled with [¹⁴C]proline, were prepared by the methods of Harwood *et al.* (1977). Other chemicals were of AnalaR grade whenever available.

Isolation and incubation of rat lens capsules

Lenses, taken from the eyes of rats killed by decapitation, were rolled on coarse paper to remove any adherent vitreous body. The capsule, together with its attached epithelium, was then peeled off the body of the lens under a dissecting-microscope, after incision with a Ziegler iridectomy knife. Tissues were kept at 4°C in modified Krebs medium (Dehm & Prockop, 1971) and collected by centrifugation at 600g for 4 min. In most experiments 10–15 capsules were preincubated for 15 min at 37°C in 0.75 ml of filter-sterilized Krebs medium containing 10% (v/v) dialysed foetal bovine serum plus penicillin G (100 i.u./ml) and streptomycin (100 µg/ml). Incubations with [5-³H]proline were continued for up to 6 h at 37°C and terminated by the addition of $\alpha\alpha'$ -bipyridine (1 mM), cycloheximide (100 µg/ml), phenylmethanesulphonyl fluoride (1.7 mM), *N*-ethylmaleimide (10 mM) and EDTA (25 mM) to the final concentrations indicated.

In pulse-chase experiments the lens capsules were

incubated for 2 h with [³H]proline followed by a further 4 h in the presence of 100 µg of the non-radioactive imino acid/ml. In experiments where the chase period was extended to 22 h the conditions were as above, except that Dulbecco's modification of Eagle's medium containing 10% (v/v) dialysed foetal bovine serum and gentamicin (50 µg/ml) was used for the incubation. When β -aminopropionitrile fumarate was included in pulse-chase experiments, it was present throughout the whole incubation period, at a concentration of 50 µg/ml.

Synthesis of collagen by isolated lens capsules

The synthesis of hydroxy[³H]proline was taken as a measure of collagen synthesis when capsules were incubated with [5-³H]proline (10 µCi/ml) at 37°C in polypropylene tubes. At the end of the incubation, further hydroxylation was inhibited by the addition of $\alpha\alpha'$ -bipyridine (Prockop, 1971), protein synthesis was stopped with cycloheximide, and proteinase inhibitors were also added to minimize the possibility of proteolysis of the newly synthesized macromolecules. Samples were then dialysed exhaustively against water containing 5 mM-EDTA and 5 mM- ϵ -amino-hexanoic acid before hydrolysis in 6 M-HCl for 24 h at 110°C. The hydrolysates were evaporated to dryness, and the 4-hydroxy[³H]proline content of each was assayed by the method of Juva & Prockop (1966).

Gel filtration of collagenous polypeptides on SDS/agarose

The molecular size of the collagenous ³H-labelled polypeptides was examined by gel filtration on SDS/agarose after reduction and alkylation. The chromatography was performed at room temperature on columns (95 cm × 1.5 cm) of 6% agarose (Bio-Gel A-5m). Descending elution was carried out at approx. 2.5 ml/h with 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.1% SDS and 0.02% NaN₃. Fractions (approx. 2 ml) were collected and a sample of each was taken for measurement of radioactivity in a scintillation fluid comprising 5 vol. of toluene containing 2,5-diphenyloxazole (7.5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.15 g/l) and 2 vol. of ethylene glycol monomethyl ether. The recovery of radioactivity from the column was consistently greater than 85%.

To the capsule incubation mixture containing the proteinase inhibitors was added [¹⁴C]proline-labelled chick tendon procollagen (50000 c.p.m. approx.), which served as an internal marker in the column chromatographic analyses. The samples were made 100 mM with respect to Tricine buffer, pH 8.3, and SDS (2%, w/v), urea (0.1 M) and dithiothreitol (50 mM) were added to the concentrations indicated. The samples were incubated at 100°C for 3 min, and then

at 37°C for 2h. Alkylation was carried out at room temperature (23°C) for 30min with 100mM-iodoacetamide and the pH was maintained at approx. 8 with 4M-NaOH, by using a drop of phenolphthalein as internal indicator. After exhaustive dialysis against the column-elution buffer, to which 1mM-EDTA had been added, the sample was centrifuged to remove insoluble material. The supernatant, containing over 95% of the peptide-bound radioactivity, was then applied to the column.

Blue Dextran 2000 was used to determine the void volume and $^3\text{H}_2\text{O}$ (approx. 0.05 μCi) the total column volume. For molecular-weight determinations the column was calibrated with a mixture of rat tail-tendon collagen α - and β -chains (mol.wts. 98000 and 196000 respectively), chick tendon pro- α -chains (mol.wt. 150000) and ovalbumin (mol.wt. 45000).

SDS/polyacrylamide-gel electrophoresis

The nature of the collagenous ^3H -labelled polypeptides synthesized by isolated lens capsules was examined by SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). The macromolecules were prepared for analysis by precipitation from the complete incubation mixture with $(\text{NH}_4)_2\text{SO}_4$ at 75% saturation in the presence of proteinase inhibitors at 4°C. The precipitates were incubated at 100°C for 2min in sample preparation buffer containing 2% (w/v) SDS, 2M-urea and 50mM-dithiothreitol. Subsequent dialysis for up to 20h against two changes of sample buffer (50ml) at room temperature promoted solubilization and removed residual $(\text{NH}_4)_2\text{SO}_4$. This procedure solubilized approx. 95% of the radioactivity in the precipitate.

Polyacrylamide gels were prepared in glass tubes (internal diameter 0.6cm) and consisted of a separating gel (10cm) containing 5% (w/v) polyacrylamide and a stacking gel (1cm) containing 3% (w/v) polyacrylamide. Electrophoresis was carried out over a period of 8–10h at room temperature at 1.5mA/gel, and the gels were stained with Coomassie Brilliant Blue R by the method of Fairbanks *et al.* (1971). Where appropriate, duplicate gels were stained with the periodic acid/Schiff reagent by sequential immersion in 50% (v/v) methanol/7% (v/v) acetic acid, 1% (w/v) periodic acid in 7% acetic acid, 7% acetic acid, Schiff's reagent and 1% (w/v) sodium metabisulphite in 0.1M-HCl. The Coomassie Blue-stained gels were frozen and sliced with a parallel array of razor blades (providing slices 1.15mm thick); each slice was digested in a closed scintillation vial containing 0.5ml of NCS/water (9:1, v/v) for 2h at 65°C and then for 16h at room temperature. Scintillation fluid [2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l) dissolved in toluene] (10ml) was added to each vial and radioactivity determined after a further incubation of 3–4 days at

room temperature. Regions corresponding to the peaks of radioactivity were removed from duplicate gels, hydrolysed (Houston, 1971) and assayed for 4-hydroxy[^3H]proline (Juva & Prockop, 1966).

Preparation of capsular basement membrane

The capsular basement membrane was prepared free of cellular material by sequential extraction of the tissue with distilled water, 1M-NaCl containing approx. 2000 Kunitz units of deoxyribonuclease (Kunitz, 1950), and 4% (w/v) sodium deoxycholate (Meezan *et al.*, 1975). This procedure has been shown to yield an insoluble residue that is morphologically and chemically similar to basement-membrane material prepared by sonication (Meezan *et al.*, 1975; J. G. Heathcote, unpublished work). To minimize degradation of newly synthesized polypeptides the extractions were performed in the presence of phenylmethanesulphonyl fluoride (1.7mm), *N*-ethylmaleimide (10mm) and EDTA (25mm), the EDTA being incorporated only after the deoxyribonuclease digestion. The duration of each extraction period was decreased to three-quarters of that recommended by Meezan *et al.* (1975). The deoxycholate-insoluble residue was analysed by SDS/polyacrylamide-gel electrophoresis as described above.

Results

Incorporation of [^3H]proline into collagenous polypeptides

When isolated lens capsules, each consisting of the capsular basement membrane and its associated epithelium, were incubated with [^3H]proline, the incorporation of label into non-diffusible polypeptides was linear with time over a period of 6h (Fig. 1). The formation of 4-hydroxy[^3H]proline was also linear over this period and approx. 4% of the incorporated radioactivity was present as 4-hydroxy[^3H]proline. A modified Krebs medium was used (Dehm & Prockop, 1971), containing 10% foetal bovine serum dialysed to remove free proline. The serum was included to minimize the proteolytic degradation of the newly synthesized macromolecules that had been observed in earlier work with short-term cell incubations (Grant *et al.*, 1972b). At the end of a 6h incubation period approx. 30% of the polypeptide-bound 4-hydroxy[^3H]proline was present in the medium. Therefore in subsequent studies of the newly synthesized collagenous polypeptides the incubation mixture was taken for gel-filtration chromatography. The macromolecules were prepared for electrophoretic analysis by precipitation from the whole incubation mixture with $(\text{NH}_4)_2\text{SO}_4$ at 75% saturation.

Fig. 1. Incorporation of [^3H]proline and synthesis of 4-hydroxy[^3H]proline by isolated rat lens capsules. Duplicate samples of five lens capsules were incubated with [^3H]proline ($10\mu\text{Ci}$) in 1 ml of modified Krebs medium containing 10% (v/v) dialysed foetal bovine serum. The incubations were stopped after an appropriate time, the samples dialysed and then hydrolysed in 6M-HCl for 24 h at 110°C . The hydrolysates were assayed for total ^3H (●) and 4-hydroxy[^3H]proline (○).

Molecular-size analysis of collagenous polypeptides synthesized by isolated lens capsules

Since basement membranes can be solubilized by treatment with SDS in the presence of a reducing agent (Hudson & Spiro, 1972a), this method was used to solubilize the macromolecules synthesized by isolated lens capsules during a 2 h incubation with [^3H]proline. The denatured polypeptides were then analysed by gel-filtration chromatography. Most of the ^3H -labelled polypeptides were eluted in positions corresponding to the mol.wt. range 12000–100000. However, a significant peak of radioactivity (18% of the total c.p.m. recovered from the column) was eluted two fractions ahead of the internal marker of type-I pro- α -chains (Fig. 2a). This component had an apparent mol.wt. of 180000 and contained all the 4-hydroxy[^3H]proline synthesized by the lens capsules during the incubation period. Approx. 40% of the radioactivity in the peak fraction was in the form of 4-hydroxy[^3H]proline, as determined by the method of Juva & Prockop (1966). The above result was obtained in three separate experiments and when collagen β -chains were also included as an internal standard, the basement-membrane collagen polypeptides were eluted one fraction after the β -chains and two fractions ahead of the tendon pro- α -chains (Fig. 3). This observation confirmed that the basement-membrane collagen polypeptides were of a molecular weight just less than collagen β -chains.

The possible conversion of the collagenous polypeptides into lower-molecular-weight species was investigated by experiments in which the 2 h incu-

bation with [^3H]proline was followed by a 4 h chase period in the presence of unlabelled proline. The samples were analysed by gel-filtration chromatography (Fig. 2b). No evidence was obtained for the presence of hydroxy[^3H]proline in a species of mol.wt. below 180000 (peak 1, Fig. 2b), suggesting that cleavage of the collagenous polypeptides did not occur under these experimental conditions. However, after the chase period of 4 h, approx. 18% of the 4-hydroxy[^3H]proline was eluted in a peak of mol.wt. considerably greater than 180000 (peak 2, Fig. 2b). Experiments on the formation of this material will be presented below.

Analyses of hydrolysates of peak 1 (fraction 43, Figs. 2a and 2b) by ion-exchange chromatography indicated that 4-hydroxy[^3H]proline accounted for approx. 47% of the radioactivity in the peak (Fig. 4). Only 2.5% of the total radioactivity was present as 3-hydroxy[^3H]proline.

Electrophoretic analysis of collagenous polypeptides

Polyacrylamide-gel electrophoresis was used to confirm the results obtained by gel-filtration chromatography. When isolated lens capsules were incubated for 2 h with [^3H]proline and then analysed on gels most of the radioactivity migrated near the dye front and was believed to include newly synthesized crystallins (Fig. 5a). There was also a prominent single peak of high-molecular-weight material, and 4-hydroxy[^3H]proline accounted for 36% of the radioactivity in this peak (peak I, Fig. 5a). This material migrated slightly faster than collagen β -chains, but more slowly than the pro- α -chains of embryonic-chick tendon and cartilage; this behaviour is consistent with a species of approx. mol.wt. 180000.

When the 2 h pulse-label was followed by a 4 h chase period there was no evidence of conversion of the initially synthesized polypeptides into species of lower molecular weight, but a peak of higher molecular size was observed (peak II, Fig. 5b). In occasional experiments a small peak of radioactivity migrating in this position was also detected after the 2 h pulse-label (Fig. 5a). The collagenous nature of both these peaks was confirmed by their susceptibility to digestion with bacterial collagenase (Fig. 6). It was therefore concluded that these components corresponded to the hydroxy[^3H]proline-containing peaks (peaks 1 and 2, Fig. 2b) obtained in the gel-chromatographic analyses. Peaks I and II had similar electrophoretic mobilities to two of the bands (bands B and A respectively) observed when the gels were stained with Coomassie Brilliant Blue (gel c, Fig. 7). Although band C appeared to be a true basement-membrane component (see below), it did not correspond to a peak of radioactivity under the conditions of these biosynthetic experiments. Bands A and B were also stained by the periodic

Fig. 2. Gel-filtration chromatography on SDS/agarose (Bio-Gel A-5m) of ^3H -labelled proteins synthesized by isolated rat lens capsules

Rat lens capsules (30) were incubated with $100 \mu\text{Ci}$ of $[^3\text{H}]$ proline in 1.5 ml of modified Krebs medium containing 10% (v/v) dialysed foetal bovine serum for (a) 2h and (b) 2h followed by a chase period of 4h in the presence of non-radioactive proline ($100 \mu\text{g/ml}$). After incubation, proteinase inhibitors were added and the whole incubation mixture was denatured in SDS, reduced with dithiothreitol and alkylated with iodoacetamide. The sample was dialysed against 0.02M-Tris/HCl buffer, pH 7.4, containing 0.1% SDS and 0.02% NaN_3 before co-chromatography with $[^{14}\text{C}]$ proline-labelled embryonic-chick tendon procollagen which had been added to the sample before denaturation. The void volume of the column was in fraction 35 (63 ml) and the total volume was in fraction 96 (173 ml). For reference purposes the peaks of 4-hydroxy $[^3\text{H}]$ proline have been designated 1 and 2, and the elution position of tendon pro- α -chains is marked T. ●, Total ^3H ; ○, 4-hydroxy $[^3\text{H}]$ proline.

acid/Schiff reagent, indicating the presence of appreciable quantities of carbohydrate.

Assembly of collagenous polypeptides into high-molecular-weight material

The experiments described above indicated that after a 2h pulse-label followed by a 4h chase approx. 18% of the 4-hydroxy $[^3\text{H}]$ proline was present in material of mol.wt. greater than 180000 (peak 2, Fig. 2b). When the pulse-chase experiments were repeated in the presence of β -aminopropionitrile the formation of the polypeptide (mol.wt. >180000) was completely abolished (results not shown), thus implicating either lysine- or hydroxylysine-derived cross-links in its assembly.

The chase period was extended from 4 to 22h in a series of experiments in which isolated lens capsules

were incubated with $[^3\text{H}]$ proline in Dulbecco's modification of Eagle's medium. Under these conditions 40% of the total 4-hydroxy $[^3\text{H}]$ proline was found in peak 2 when the total incubation mixture was analysed by gel-filtration chromatography (Fig. 8b). The presence of β -aminopropionitrile during the 2h pulse and 22h chase decreased this value to about 10% (Fig. 8c). The results of a typical experiment are presented in Table 1; they suggest that, with time, there had been a net transfer of hydroxyproline-containing material from peak 1 to peak 2.

Incorporation of newly synthesized collagenous polypeptides into the lens capsular basement membrane

To confirm that the collagenous polypeptides of apparent mol.wt. 180000 were components of base-

Fig. 3. Comparison of the size of lens-capsule collagen precursor polypeptides with type-I collagen subunits by SDS/agarose gel-filtration chromatography

Lens capsules (11) were incubated with [^3H]proline (25 μCi) for 2 h in 0.75 ml of modified Krebs medium containing 10% (v/v) dialysed foetal bovine serum. The incubation was stopped and proteinase inhibitors were added. To the complete incubation mixture were added rat tail-tendon collagen (5 mg) and [^{14}C]proline-labelled embryonic-chick tendon procollagen (approx. 50000 c.p.m.) and the sample was prepared for chromatography as described in the text. A sample (0.2 ml) of each fraction was assayed for total ^3H (●) and total ^{14}C (■), and the A_{230} (□) of each fraction was also determined. A portion of the elution profile is shown and the elution position of collagen β -chains is indicated by the arrow.

Fig. 4. Determination by ion-exchange chromatography of the proportions of 3-hydroxyproline and 4-hydroxyproline in newly synthesized lens-capsule collagen precursor

Fraction 43 (Fig. 2b) was hydrolysed in 6M-HCl at 110°C for 24 h in a sealed tube. The hydrolysate was evaporated to dryness and dissolved in 1.5 ml of 7 mM-sodium citrate buffer, pH 3.0. A sample (1.0 ml) was applied to the long column of a JEOL JLC 6AH automatic amino acid analyser, which was eluted with the pH 3.0 buffer. A sample (0.5 ml) of each fraction (0.75 ml) was counted in 10 ml of scintillation fluid comprising 2 vol. of toluene containing 2,5-diphenyl-oxazole (5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1 g/l) and 1 vol. of Triton X-100. The elution positions of 3-hydroxyproline (1), 4-hydroxyproline (2) and proline (3) are indicated.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of ^3H -labelled polypeptides synthesized by isolated rat lens capsules. In duplicate experiments 12 capsules were incubated with ^3H proline ($25\ \mu\text{Ci}$) in 0.75 ml of modified Krebs medium containing 10% (v/v) dialysed foetal bovine serum for (a) 2 h and (b) 2 h, followed by a 4 h chase period in the presence of non-radioactive proline ($100\ \mu\text{g/ml}$). Samples were denatured, reduced and electrophoresed as described in the text. The mobilities of the following standards are indicated: 1, collagen β -chains (β_{11}); 2, collagen β -chains (β_{12}); 3, chick tendon procollagen pro- α_1 -chains; 4, chick tendon procollagen pro- α_2 -chains; 5, collagen α_1 -chains; 6, collagen α_2 -chains. The position of the Bromophenol Blue tracking dye is indicated by the heavy arrow.

ment membrane, radioactively labelled capsular basement membrane was prepared by a modification of the method of Meezan *et al.* (1975). Isolated lens capsules were incubated for 24 h with ^3H proline in Dulbecco's modification of Eagle's medium and the medium was removed after centrifugation. The capsules were then extracted with sodium deoxycholate as described above and the insoluble residue was examined by SDS/polyacrylamide-gel electrophoresis. A major peak of radioactivity migrated slightly faster than the β -chains of rat tail-tendon collagen (Fig. 9) and had an electrophoretic mobility similar to peak I (Fig. 5a). This peak also corresponded to a major Coomassie Blue-positive band (band B, gel d, Fig. 7). The Coomassie Blue-staining pattern obtained with the deoxycholate-insoluble basement membrane showed three major bands (bands A, B and C, gel d, Fig. 7) and was similar to the pattern obtained with the intact isolated lens capsule, except for the absence of lower-molecular-weight bands corresponding to cellular proteins (gel c, Fig. 7). It was not possible to demonstrate peaks of radioactivity corresponding to bands A and C in the deoxycholate-insoluble basement membrane.

Discussion

The general insolubility of basement membranes has hampered the characterization of their subunits, although limited proteolytic digestion of the membranes with pepsin has permitted the isolation of a number of collagenous polypeptides. The efficacy of this technique is usually ascribed to digestion of the non-helical extremities of the collagen molecules where the stabilizing cross-links are found (Miller, 1976). However, the assumption that the triple-helical portion of the collagen molecule is resistant to proteolysis may not be valid for all collagen types, since it has been shown that type-III collagen is susceptible to trypsin at a particular locus within the helix (Miller *et al.*, 1976). If basement-membrane collagen exhibited similar susceptibility to proteolysis, as has been suggested (Schwartz & Veis, 1978; Timpl *et al.*, 1978), this might account for the different sizes of the collagenous polypeptides isolated from pepsin digests of various basement membranes (Kefalides, 1971, 1973; Daniels & Chu, 1975; Burgeson *et al.*, 1976; Dixit, 1978).

No detailed analysis of lens-capsule polypeptides obtained by direct solubilization procedures has been

Fig. 6. Effect of bacterial collagenase on the high-molecular-weight polypeptides synthesized by isolated rat lens capsules. In duplicate experiments 12 lens capsules were incubated with $[^3\text{H}]$ proline ($25\ \mu\text{Ci}$) for 6 h in 0.75 ml of Dulbecco's modification of Eagle's medium. The incubation was stopped and proteinase inhibitors were added as described in the text, except that EDTA was omitted. The incubation mixtures were dialysed exhaustively at 4°C against water containing 10 mM- ϵ -aminohexanoic acid and then against 200 ml of 62.5 mM-Tris/HCl buffer (pH 7.4) containing 5 mM- CaCl_2 , 2 mM- N -ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride. One sample was treated with highly purified bacterial collagenase (enzyme/substrate ratio of approx. 1:100, w/w) at 37°C for 2 h and the other was incubated without the enzyme. Equal amounts of each sample were subjected to SDS/polyacrylamide-gel electrophoresis as described in the text. A sample of rat tail-tendon collagen was treated in the same way to check the activity of the enzyme. The mobilities of the following standards are indicated: 1, collagen β -chains (β_{11}); 2, collagen β -chains (β_{12}); 3, collagen α_1 -chains; 4, collagen α_2 -chains. The position of the Bromophenol Blue tracking dye is indicated by the heavy arrow. —, Control sample; ----, digested sample.

reported. Olsen *et al.* (1973) presented electron micrographs of a citrate extract of sheep anterior-lens capsule, which showed macromolecules with both collagenous and non-collagenous domains. However, citrate buffers only solubilize approx. 10% (by weight) of this basement membrane (Kefalides, 1973), whereas the combination of a reducing agent with a denaturant such as SDS will solubilize up to 80% of both lens capsule and glomerular basement membrane (Hudson & Spiro, 1972a; Kefalides, 1973). Accordingly, in this study we have attempted to correlate the newly synthesized collagenous polypeptides of the rat lens capsule with the components solubilized from intact lens capsules by SDS in the presence of dithiothreitol.

In short-term incubations of isolated rat lens capsules incorporation of $[^3\text{H}]$ proline was linear with respect to time (Fig. 1), but only a small percentage of the $[^3\text{H}]$ proline became hydroxylated. Similar results were obtained in earlier experiments with chick lens cells (Grant *et al.*, 1972a) and isolated rat renal glomeruli (Grant *et al.*, 1975). When the

^3H -labelled polypeptides synthesized during a 2 h incubation were analysed by gel-filtration chromatography under reducing conditions, all the 4-hydroxy- $[^3\text{H}]$ proline was found in a single high-molecular-weight peak (peak 1, Fig. 2a) that was quite distinct from the remainder of the labelled polypeptides. This species was estimated to have an apparent mol.wt. of 180000, i.e. just less than that of the β -components of rat tail-tendon collagen (Fig. 3), and its electrophoretic mobility in SDS/polyacrylamide gels was compatible with such a size (Fig. 5). The presence of a single symmetrical peak on SDS/polyacrylamide-gel electrophoresis suggested that only one type of polypeptide chain was present in the newly synthesized molecule.

Approx. 47% of the radioactivity in the peak fraction of interest (fraction 43, Figs. 2a and 2b) was accounted for by 4-hydroxy $[^3\text{H}]$ proline, whereas in the α -chain (mol.wt. 109000) of type-IV collagen from sheep anterior-lens capsule approx. 70% of the imino acid content was in the form of 4-hydroxyproline (Kefalides, 1971). This observation suggests

Fig. 7. Diagrammatic representation of the electrophoretic analysis of the polypeptides of isolated rat lens capsules

Lens capsules (9) with attached epithelium were solubilized in electrophoresis sample buffer, electrophoresed in SDS/polyacrylamide gels and stained with Coomassie Brilliant Blue R as described in the text. Deoxycholate-insoluble lens-capsular material, prepared by the method of Meezan *et al.* (1975), was treated in a similar way and both samples were compared with standards of rat tail-tendon collagen and chick tendon procollagen. (a) Rat tail-tendon collagen; (b) chick tendon procollagen; (c) isolated rat lens capsules; (d) deoxycholate-insoluble lens capsules. The major Coomassie Blue-staining bands have been designated A, B and C for reference purposes (see the Discussion section) and the position of the Bromophenol Blue tracking dye is indicated by the arrow.

that the peak fraction contained some non-collagenous polypeptide material. The precursor polypeptides of interstitial collagens carry non-collagenous peptide extensions at both their *N*- and *C*-termini (for review see Grant & Jackson, 1976) and it is therefore possible that the species of mol.wt. 180000 may represent the pro- α -chain of basement-membrane procollagen. Since there is no definite evidence for a structure similar to that of pro- α -chains in this species, it is hereafter referred to as the basement-membrane collagen precursor. Other published values for the size of the precursor are smaller than the one presented here. The early studies on the chick lens suggested a mol.wt. of 140000 (Grant *et al.*, 1972b) and more recent work by Kefalides and his colleagues on parietal yolk-sac endoderm (Minor *et al.*, 1976a), chick lenses (Minor *et al.*, 1976a), corneal endothelium (Kefalides *et al.*, 1976) and vascular endothelial cells (Howard *et al.*, 1976) has indicated a mol.wt. in the range 135000–160000. Orkin *et al.* (1977) have described a basement-

membrane collagen of mol.wt. 185000 that was synthesized by mouse teratocarcinoma cells in culture. The variation in observed molecular size may be the result of proteolytic degradation in some of these systems (Minor *et al.*, 1976a; Kefalides *et al.*, 1976) despite the use of proteinase inhibitors during the analytical procedures. In short-term cultures (up to 24h) of isolated rat lens capsules there was no evidence of degradation of the newly synthesized basement-membrane collagen precursor (Fig. 8b).

Preliminary characterization of the basement-membrane collagen precursor indicated that approx. 5% of its hydroxyproline residues occurred as the 3-isomer (Fig. 4). Kefalides (1973) has emphasized the importance of 3-hydroxyproline as a marker for the type-IV collagen of basement membranes, which contains 10–23 residues of 3-hydroxyproline per 1000 amino acid residues. However, collagens that contain much lower amounts of 3-hydroxyproline have been isolated from lens capsule (Trelstad & Kang, 1974; Dixit, 1978), and Lembach *et al.* (1977) have shown that human skin fibroblasts in culture are able to synthesize a collagen (presumed to be type I) containing unexpectedly large amounts of 3-hydroxyproline during the phase of attachment to the flask. The real value of 3-hydroxyproline as a marker for basement-membrane collagen will remain uncertain until there is more information on the metabolism and function of this imino acid.

The procollagens of the interstitial connective tissues are converted by specific extracellular enzymes into their respective collagen fibril monomers (for review see Grant & Jackson, 1976). Initially it was thought that the basement-membrane collagen precursor underwent a similar modification (Grant *et al.*, 1972b, 1975), but when proteinase inhibitors were present during the analysis of the precursors synthesized by parietal yolk-sac endoderm and chick lenses this conversion was not observed (Minor *et al.*, 1976a). In the experiments reported here there was no evidence of conversion of the lens-capsule collagen precursor into a lower-molecular-weight species. This lack of conversion could theoretically be the result of release of the precursor into the medium where there might be a deficiency of the enzymes required to remove the peptide extensions. However, this explanation seems unlikely, since our studies demonstrate that conversion does not occur even during a prolonged (22h) chase period (Fig. 8b) and that the newly synthesized precursor appears to be incorporated directly into the membrane (Fig. 9) which contains a major polypeptide subunit of similar molecular weight (band B, gel d, Fig. 7). Also we have found no evidence for conversion of the glomerular basement-membrane collagen precursor, which is not released into the incubation medium (Heathcote *et al.*, 1978). It is noteworthy that polypeptide analysis of solubilized rat lens capsule revealed

Fig. 8. Gel-filtration chromatography on SDS/agarose of ^3H -labelled proteins synthesized by isolated rat lens capsules in the presence and in the absence of β -aminopropionitrile

In three separate experiments 17 lens capsules were incubated for 2h with ^3H proline ($50\mu\text{Ci}$) in 0.9 ml of Dulbecco's modification of Eagle's medium containing 10% (v/v) dialysed foetal bovine serum. In (a) the incubation was terminated after 2h and the sample denatured, reduced and alkylated before chromatography. In (b) non-radioactive proline ($100\mu\text{g/ml}$) was added after 2h, and the incubation continued for a further 22h before analysis as above; (c) was performed in a similar manner, except that β -aminopropionitrile ($50\mu\text{g/ml}$) was present throughout the incubation. The void volume of the column was in fraction 32 (64ml) and the total volume was in fraction 91 (182ml). The elution position of tendon pro- α -chains is marked T. ●, Total ^3H ; ○, 4-hydroxy ^3H proline.

Table 1. Influence of β -aminopropionitrile on the time-dependent transfer of the basement-membrane collagen precursor into a larger basement-membrane component

The experimental conditions were identical with those described in the legend to Fig. 8, in which lens capsules were incubated with ^3H proline for 2h and the radioactivity was chased with unlabelled proline for 22h.

Expt.	4-Hydroxy ^3H proline content (d.p.m.)		Proportion of total 4-hydroxy ^3H proline in Peak 2
	Peak 1	Peak 2	
(a) 2h pulse	65863	0	0
(b) 2h pulse, 22h chase; without β -aminopropionitrile	32293	24417	43
(c) 2h pulse, 22h chase; with β -aminopropionitrile	54260	5902	10

Fig. 9. SDS/polyacrylamide-gel electrophoresis of ^3H -labelled polypeptides incorporated into deoxycholate-insoluble lens capsule

Lens capsules (37) were incubated with ^3H proline ($150\mu\text{Ci}$) for 24 h in 3 ml of Dulbecco's modification of Eagle's medium containing 10% (v/v) dialysed foetal bovine serum. The incubation was stopped and proteinase inhibitors were added as described in the text. The medium was removed and the capsules were freed of cellular components by the method of Meezan *et al.* (1975). The washed deoxycholate-insoluble basement membrane was solubilized in electrophoresis sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis. The mobilities of the following standards are indicated: 1, collagen β -chains (β_{11}); 2, collagen β -chains (β_{12}); 3, chick tendon procollagen pro- α_1 -chains; 4, chick tendon procollagen pro- α_2 -chains; 5, collagen α_1 -chains; 6, collagen α_2 -chains.

a component in the mol.wt. range 120000–150000 (band C, gels *c* and *d*, Fig. 7), the nature of which has not yet been determined. This component could represent a derivative of the collagen precursor, but, since it did not become labelled under the conditions of these experiments, the processing must occur after a considerable delay and after the precursor has become integrated within the membrane.

The incorporation of radioactive amino acids into basement membranes has been demonstrated several times by both radioautographic and chemical methods (see, e.g., Lazarow & Speidel, 1964; Young & Ocumpaugh, 1966; Minor *et al.*, 1976*b*; Hjelle *et al.*, 1976; Price & Spiro, 1977). However, our observation that the basement-membrane collagen precursor is incorporated directly into the lens capsule (Fig. 9) represents the first clear demonstration of the biosynthesis of a defined basement-membrane subunit and its subsequent incorporation into the membrane. The method of preparation of basement membrane (Meezan *et al.*, 1975) is protracted and may predispose towards degradation of the basement-membrane components. Despite the inclusion of proteinase inhibitors in the analytical procedure the

Coomassie Blue-positive bands on the gels tended to be broad (gel *d*, Fig. 7) and the profile of radioactivity had an irregular baseline (Fig. 9), suggesting that some degradation had occurred. Such degradation might also account for the failure to demonstrate a peak of radioactivity in the deoxycholate-insoluble material of similar electrophoretic mobility to band A (gel *d*, Fig. 7).

However, the biosynthesis of a component corresponding to band A (gels *c* and *d*, Fig. 7) was demonstrated in the pulse-chase experiments described in Figs. 2*b*, 5*b* and 8*b*. The nature of this material is uncertain, although it is susceptible to digestion by purified bacterial collagenase (Fig. 6) and it may represent a complex of the basement-membrane collagen precursor with another protein. If this were the case, the linkage involved must be something other than disulphide bonds, since both the gel-filtration chromatography and the polyacrylamide-gel electrophoresis were performed under reducing conditions. Since β -aminopropionitrile almost abolished the formation of this material (Fig. 8*c* and Table 1) it would appear to be cross-linked by lysine- or hydroxylysine-derived bonds

(for review see Bailey *et al.*, 1974). It has been suggested that hydroxylysine-derived cross-links are present in basement membranes in significant amounts (Tanzer & Kefalides, 1973; Minor *et al.*, 1975), though Sato & Spiro (1972) found negligible quantities in bovine glomerular basement membrane. There is no information on the functions of such cross-links in basement membranes, although they may have a role in stabilizing the association between subunits. For a better understanding of the assembly of basement membranes a more precise picture of the molecular nature of the collagenous components will be required.

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