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Studies on the assembly of the rat lens capsule

BIOSYNTHESIS OF A CROSS-LINKED COLLAGENOUS COMPONENT OF HIGH MOLECULAR WEIGHT

J. Godfrey HEATHCOTE,* Allen J. BAILEY† and Michael E. GRANT*
**Department of Medical Biochemistry, University of Manchester Medical School, Oxford Road, Manchester M13 9PT, U.K., and †A.R.C. Meat Research Institute, Langford, Bristol BS18 7DY, U.K.*

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1. Intact rat lenses in tissue culture synthesize hydroxy[^{3}H]proline-containing polypeptides of apparent mol.wt. approx. 180000, which become assembled into aggregates of higher molecular weight with time. 2. Both the 180000-mol.wt. species and the aggregates are components of the deoxycholate-insoluble basement-membrane matrix. 3. Formation of the high-molecular-weight aggregate is accompanied by the biosynthesis of the reducible hydroxylysine-derived cross-link hydroxylysino-5-oxonorleucine. 4. Hydroxylysino-5-oxonorleucine and dehydrohydroxylysinonorleucine are the major reducible cross-links present in intact foetal and 1-month-old calf lens capsules.

Despite intensive biochemical investigation of basement membranes, there is no general agreement on the molecular form of their collagenous component (for review see Kefalides *et al.*, 1979). Biosynthetic studies *in vitro* indicate that basement-membrane collagen resembles the procollagens of interstitial connective tissues in structure (for review see Grant *et al.*, 1979). Such biosynthetic studies have largely concentrated on characterization of the newly synthesized collagen molecules released into the incubation medium (see, e.g., Howard *et al.*, 1976; Kefalides *et al.*, 1976), and the precise fate of these polypeptides has not been established. Only for rat lens capsule has it been clearly demonstrated that these molecules represent true components of the basement-membrane matrix (Heathcote *et al.*, 1978).

Analytical studies suggest that basement membranes contain other glycoproteins besides collagen, but the interaction of these two components has not been investigated. The mode of assembly of basement membranes from the collagen and glycoprotein subunits is central to an understanding of the organization and function of these structures, and the identity of the intermolecular bonds that stabilize the matrix is of particular interest in this connection. It is clear from the work of Hudson & Spiro (1972*a*) and Kefalides (1973) that disulphide bonds are of great importance, although their exact location has not been defined. The significance of hydrophobic bonds is more difficult to evaluate, although they have been implicated in the interaction of collagen with glycoprotein in the aortic tunica media (Moczar *et al.*, 1977). Lysine- and/or hydroxylysine-derived cross-links are found in fibrillar collagen (for reviews see Bailey *et al.*, 1974; Stimler & Tanzer, 1977), and have also been identified in basement membranes such as lens capsule and glomerular basement membrane (Tanzer & Kefalides, 1973). In the present paper we give evidence that the formation of the reducible cross-link hydroxylysino-5-oxonorleucine is an important step in the assembly of the rat lens capsule. For purposes of comparison the cross-links of intact bovine lens capsules have also been examined.

Abbreviation used: 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, Earle's balanced salt solution, foetal bovine serum and gentamycin were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and BME vitamins (100× concentrated solution) were from Gibco Bio-Cult, Hounslow, Middx., U.K. L-[5-^{3}H]Proline (23 Ci/mmol), L-[U-^{14}C]proline (275 mCi/mmol), L-[4,5-^{3}H]lysine monohydrochloride (97.5 Ci/mmol) and ^3H_2O (5 Ci/ml) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Acryl-

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amide, *NN'*-methylenebisacrylamide, Bio-Gel A-5m and Bio-Gel A-15m were obtained from Bio-Rad Laboratories, Watford, Herts., U.K. D-Penicillamine and L-amino acids were bought from Sigma (London) Chemical Co., Poole, Dorset, U.K. Embryonic-chick tendon procollagen was prepared by the method of Harwood *et al.* (1977).

Incubation of rat lenses

Intact lenses were rolled on coarse paper to remove adherent vitreous body and kept at 4°C in modified Krebs medium (Dehm & Prockop, 1971). Approx. 20 lenses were incubated at 37°C in 5ml of Dulbecco's modification of Eagle's medium containing 10% (v/v) dialysed foetal bovine serum and gentamycin (50μg/ml), referred to below as DME medium, that had been gassed with air/CO_2 (19:1). After a 15min preincubation [^{3}H]proline (200–500μCi) was added and the incubation was continued for up to 12h. In pulse–chase experiments the lenses were incubated for 6–12h with [^{3}H]proline; the lenses were then washed with fresh medium and the incubation was continued for the appropriate period in DME medium containing cycloheximide (100μg/ml) and non-radioactive proline (100μg/ml). In prolonged chase periods the medium was changed every 24h. Incubations were terminated by the addition of $\alpha\alpha'$-bipyridine (1mM), cycloheximide (100μg/ml), phenylmethanesulphonyl fluoride (1.7mM), *N*-ethylmaleimide (10mM) and EDTA (25mM) to the final concentrations indicated.

A lysine-free DME medium was prepared from the basic ingredients for experiments designed to demonstrate the biosynthesis of the cross-link.

Gel filtration of collagenous polypeptides on SDS/agarose

This procedure was performed as described previously (Heathcote *et al.*, 1978) on columns of 6% (Bio-Gel A-5m) and 4% (Bio-Gel A-15m) agarose. Fractions (approx. 2.0ml) were collected and a portion (0.2ml) of each was taken for measurement of radioactivity in a scintillation fluid comprising 5vol. of toluene containing 2,5-diphenyloxazole (7.5g/litre) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.15g/litre) and 2vol. of methoxyethanol. Where appropriate the remainder of the fraction was hydrolysed in 6M-HCl for 24h at 110°C and assayed for 4-hydroxy[^{3}H]proline by the method of Juva & Prockop (1966).

SDS/polyacrylamide-gel electrophoresis

The ^{3}H-labelled polypeptides synthesized by intact lenses were examined by slab-gel electrophoresis by the method of Laemmli (1970). Radioactively labelled lens capsules were incubated at 100°C for 2min in sample-preparation buffer containing 2% (w/v) SDS, 2M-urea and 50mM-dithiothreitol, followed by dialysis against two changes of sample buffer (50ml) at room temperature for 2h. Electrophoresis was carried out by using a separating gel containing 5% (w/v) polyacrylamide and a stacking gel containing 3% (w/v) polyacrylamide, at room temperature with a constant current of 16mA/slab gel. After fixation in 25% (v/v) propan-2-ol/10% (v/v) acetic acid, the gel was permeated with dimethyl sulphoxide and 2,5-diphenyloxazole, dried under vacuum (Bonner & Laskey, 1974) and then exposed to X-Omat XH1 X-ray film (Kodak) that had been 'pre-flashed' to increase the sensitivity of the fluorography (Laskey & Mills, 1975). Appropriate fluorograms were scanned with a Joyce–Loebl Autodensidater Mk. 3 (Cheah *et al.*, 1979).

Preparation of capsular basement membrane

The capsular basement membrane was cleansed of cellular material by a modification (Heathcote *et al.*, 1978) of the deoxycholate extraction procedure of Meezan *et al.* (1975). The deoxycholate-insoluble basement membrane was analysed by SDS/agarose gel-filtration chromatography after denaturation, reduction and alkylation, which solubilized over 90% of the radioactivity associated with the purified basement membrane.

Identification of reducible cross-links

(*a*) *Intact lens-capsule collagen.* The lens capsules from foetal calves, 1-month-old calves and 12-month-old steers were washed in saline (0.9% NaCl, pH7.4), homogenized and then reduced with KB^3H_4 as previously described (Robins *et al.*, 1973). Briefly, the reduced membranes were dialysed, freeze-dried and hydrolysed with 6M-HCl, and the amino acids were separated with a Technicon AutoAnalyzer adapted for use with volatile buffers. Confirmation of the identity of the radioactive peaks as the known intermolecular cross-links was achieved by comparison with authentic samples on the JEOL amino acid analyser.

(*b*) *[^{3}H]Lysine-labelled rat lens capsules.* The labelled lens capsules were dialysed extensively against saline to remove the excess of [^{3}H]lysine before reduction with non-radioactive KBH_4. The reduced capsules were then hydrolysed and analysed for reducible cross-links as described above.

(*c*) *Pepsin-solubilized lens-capsule collagen.* Lens capsules obtained from 1-month-old calves were washed in saline, immersed in 0.5M-acetic acid and digested with pepsin (substrate/enzyme ratio 10:1, w/w) for only 5h at 5°C. The collagen was precipitated from the acid solution with 0.9% NaCl, redissolved in 1M-NaCl/50mM-Tris/HCl buffer, pH7.4, at 4°C and re-precipitated on adjustment of the salt concentration to 4M-NaCl (Bailey *et al.*, 1979). The precipitate obtained was then reduced

with KB^3H_4 and analysed for cross-links exactly as described above.

(*d*) *Periodate oxidation.* The ^3H-labelled component eluted from the long basic column of the JEOL analyser in the position of known reduced cross-links was subjected to periodate oxidation. The component in 0.1 M-sodium citrate buffer, pH 5.3 (1 ml), was treated with 10 mM-$NaIO_4$ (1 ml) at 20°C for 5 min. The reaction was stopped and the products were reduced by adding 4 M-NaOH and KBH_4 (2.0 mg). After 30 min the solution was adjusted to pH 2.0 by the addition of 2 M-HCl, and the solution was analysed for [^3H]proline and [^3H]lysine on the JEOL analyser (Robins & Bailey, 1975).

Results

Formation of collagenous species of high molecular weight

In a previous study (Heathcote *et al.*, 1978) it was shown that the collagenous polypeptides initially synthesized by isolated rat lens capsules had an apparent mol.wt. of 180000 when analysed by gel-filtration chromatography after denaturation, reduction and alkylation. With time, these polypeptides were assembled into material of higher molecular weight, and this could be prevented by β-aminopropionitrile, indicating that lysine- or hydroxylysine-derived cross-links were involved in the assembly process. Similar biosynthetic studies have now been carried out with intact lenses, the capsules only being isolated after the incubation. After a 6 h pulse with [^3H]proline, 88% of the 4-hydroxy[^3H]proline was present in the basement-membrane collagen precursor of mol.wt. approx. 180000 (peak 1, Fig. 1*a*) and the remainder was eluted at the void volume of the column. When the 6 h pulse-label was followed by an 18 h chase period, 45% of the 4-hydroxy[^3H]proline was eluted at the void volume (peak 2, Fig. 1*b*). There was no evidence of the conversion of the precursor into a smaller molecular species.

That these two collagenous species are genuine components of the lens capsule was established by chromatographic analysis of radioactively labelled basement membrane that had been freed of cellular contamination by a modification of the deoxycholate extraction procedure described by Meezan *et al.* (1975). Intact lenses were incubated for 6 h with [^3H]proline and then for a further 18 h in the presence of non-radioactive proline. The ^3H-labelled capsules were peeled off the lenses, cleaned and prepared for gel-filtration chromatography as described previously (Heathcote *et al.*, 1978). Most of the newly synthesized polypeptides of relatively low molecular weight were removed by the purification procedure, and both the collagenous precursor and its cross-linked product were present in the preparation (Fig. 2). The hydroxy[^3H]proline peak of the precursor did not coincide with a [^3H]proline peak, and this might reflect the presence of more than one polypeptide species in this region of the chromatogram.

The nature of the cross-linked product cannot be deduced with certainty from the above pulse–chase studies. Nevertheless it appears likely that this material represents a polymer of the basement-membrane collagen precursor, since in the pulse–chase experiments the percentage hydroxylation of proline in the precursor peak (46%) and that in the void-volume peak (47%) were similar. (Each value represents the average of four determinations.) Gel-electrophoretic studies (Heathcote *et al.*, 1978) suggest that some of the cross-linked product may be in the dimeric state.

Formation of hydroxylysino-5-oxonorleucine

In experiments designed to identify the nature of the cross-link, intact lenses were incubated with [4,5-^3H]lysine in lysine-free DME medium. The capsules were peeled off and frozen at −20°C before borohydride reduction and acid hydrolysis. The only significant radioactive peak obtained from the reduced acid-hydrolysed capsules was eluted from the ion-exchange columns with pyridine/formate buffers in the position of dihydroxylysinonorleucine (peak 1, Fig. 3*a*). No significant amount of radioactivity was detectable in the known positions of the hexosyl-lysines, hydroxylysinonorleucine or histidinohydroxymerodesmosine. When the fractions containing peak 1 (Fig. 3*a*) were chromatographed on the long basic JEOL column the radioactivity was eluted in the position expected for dihydroxylysinonorleucine (Fig. 3*b*). After periodate oxidation of this component, the radioactivity was eluted in the position of proline on the acid column of the JEOL analyser (Fig. 3*c*). This result is consistent with the identification of peak 1 as dihydroxylysinonorleucine, as shown in Scheme 1.

Furthermore, the formation of cross-linked polymers was not inhibited by the presence of 1 mM-D-penicillamine in the incubation medium (results not shown), and when radioactively labelled lens capsules were extracted with 0.5 M-acetic acid at 4°C the high-molecular-weight material remained in the acetic acid-insoluble residue (results not shown). This stability to penicillamine treatment and low pH provides further support for the identification of the cross-link as hydroxylysino-5-oxonorleucine (Bailey *et al.*, 1974).

Borohydride reduction of intact bovine lens capsule

The distribution of radioactive components obtained on chromatography of the acid hydrolysate of KB^3H_4-reduced intact lens capsules revealed a

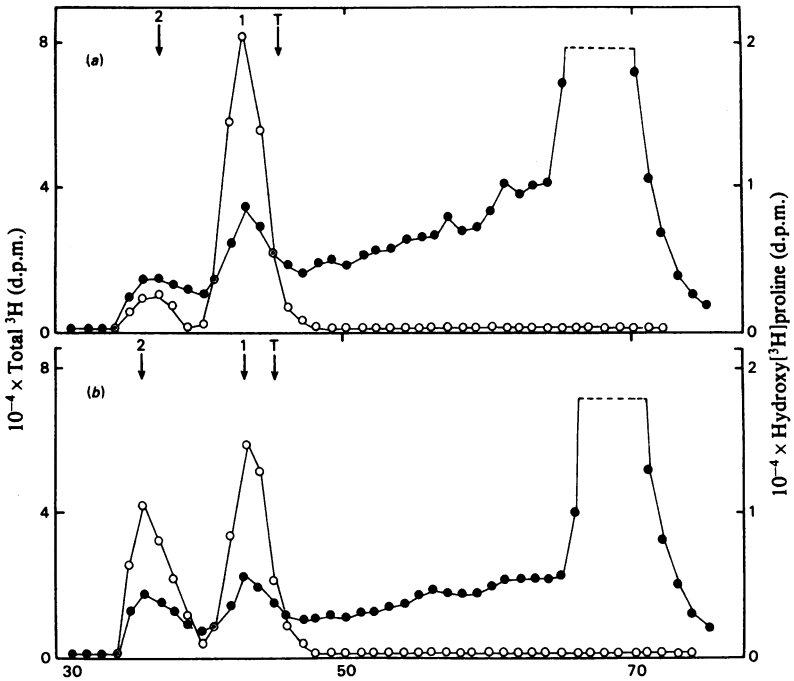


Fig. 1. *Gel-filtration chromatography on SDS/agarose (Bio-Gel A-5 m) of ^{3}H-labelled proteins synthesized by intact rat lenses*

Intact lenses (19) were incubated with [^{3}H]proline (200μCi) in 5ml of DME medium for (*a*) 6h and (*b*) 6h followed by a chase period of 18h in the presence of non-radioactive proline (100μg/ml) and cycloheximide (100μg/ml). After the incubation, proteinase inhibitors were added to the mixture and the capsules were peeled off the lenses. The capsular proteins were denatured in SDS, reduced and alkylated, and dialysed against 20mM-Tris/HCl buffer, pH7.4, containing 0.1% SDS and 0.02% NaN_3 before co-chromatography with [^{14}C]proline-labelled embryonic-chick tendon procollagen. The void volume of the column was in fraction 36 (67ml) and the total volume in fraction 96 (178ml). For reference purposes the peaks of 4-hydroxy[^{3}H]proline are numbered 1 and 2 and the elution position of chick tendon pro-α-chains is marked T. ●, Total ^{3}H; ○, 4-hydroxy[^{3}H]proline.

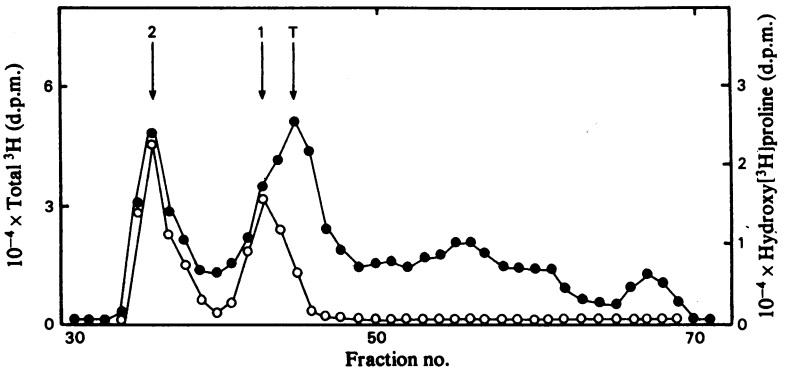
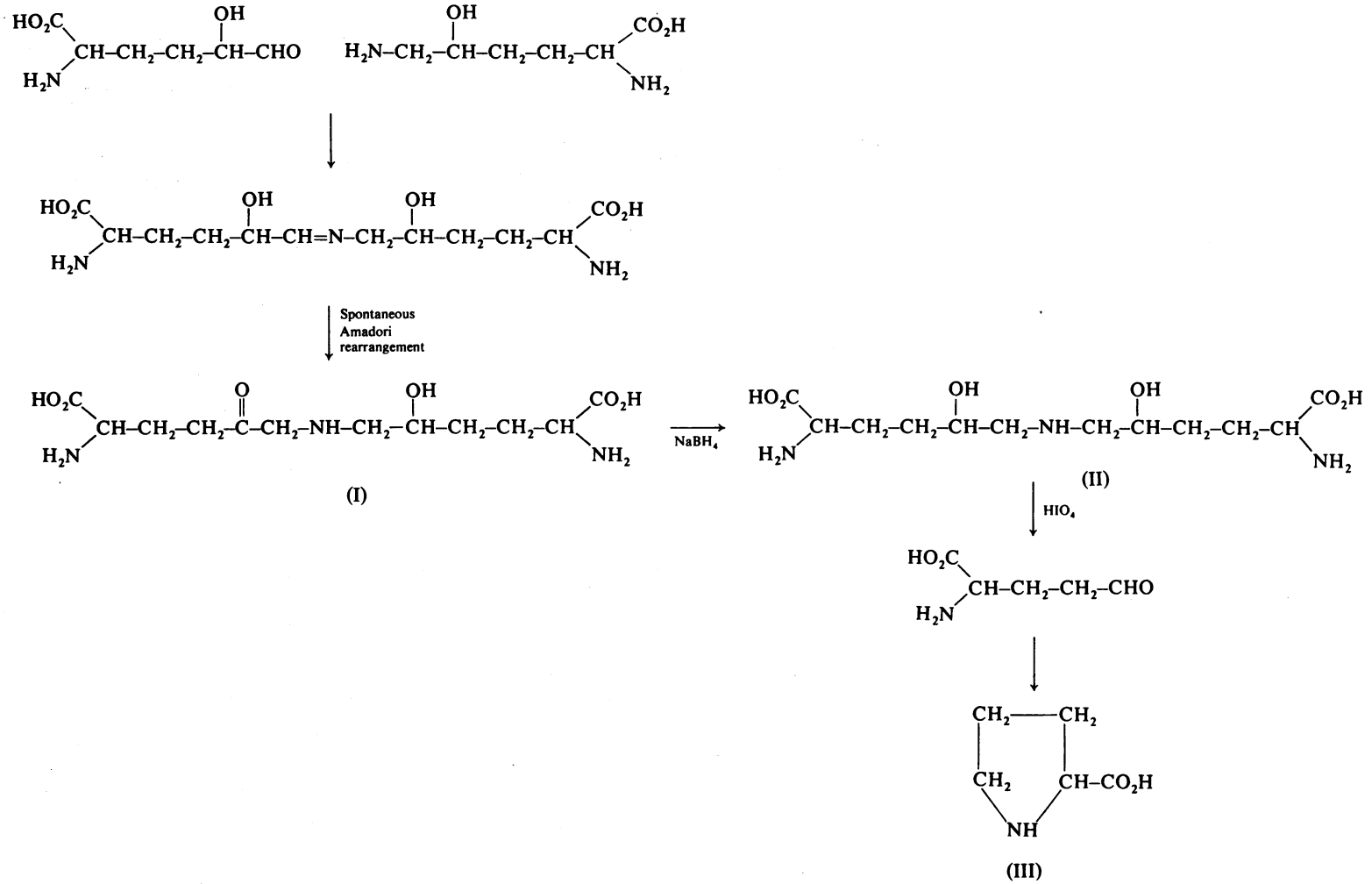


Fig. 2. *Gel-filtration chromatography on SDS/agarose (Bio-Gel A-5 m) of ^{3}H-labelled polypeptides incorporated into deoxycholate-insoluble rat lens capsule*

Intact lenses (38) were incubated with [^{3}H]proline (500μCi) in 10ml of DME medium and then incubated for a further 18h in 20ml of DME medium containing non-radioactive proline (100μg/ml) and cycloheximide (100μg/ml). At the end of the incubation, proteinase inhibitors were added, the capsules were peeled off the lenses and cleaned by the deoxycholate extraction procedure of Meezan *et al.* (1975). The deoxycholate-insoluble residue was denatured, reduced and alkylated, and co-chromatographed with embryonic-chick tendon procollagen. The peaks of 4-hydroxy[^{3}H]proline are numbered 1 and 2, and the elution position of tendon pro-α-chains is marked T. ●, Total ^{3}H; ○, 4-hydroxy[^{3}H]proline.



Scheme 1. *Biosynthesis of the cross-link hydroxylysino-5-oxonorleucine (I), formation of dihydroxylysinonorleucine (II) by sodium borohydride reduction and production of proline (III) by periodate oxidation*

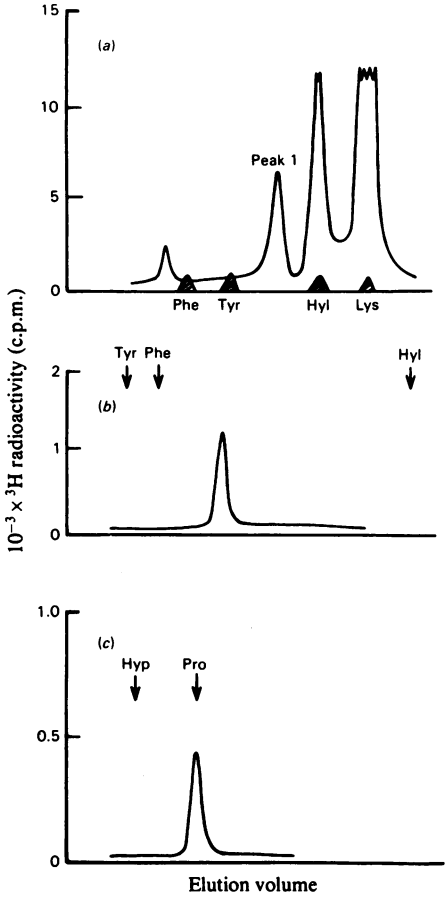


Fig. 3. *Identification of the reduced cross-link synthesized in rat lens capsule during incubation in vitro with [^{3}H]lysine*

Intact rat lenses (38) were incubated with [4,5-^{3}H]lysine (5 mCi) in 20 ml of lysine-free DME medium for 24 h. The incubation was terminated by the addition of proteinase inhibitors, and the capsules were peeled off the lenses and reduced with borohydride as described in the text. (*a*) Elution chromatogram with formic acid/pyridine buffers on the Technicon AutoAnalyzer. (*b*) Elution chromatogram of peak 1 from (*a*) by using the long basic column of the JEOL analyser and citrate buffers. (*c*) Product of periodate oxidation of peak from (*b*) eluted from the acid column of the JEOL analyser. The hatched areas indicate the elution positions of ninhydrin-positive amino acids in the hydrolysate of lens capsule.

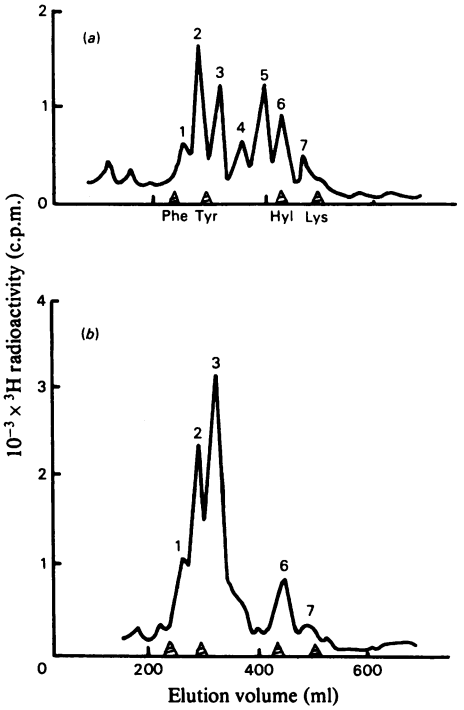


Fig. 4. *Reducible cross-links present in bovine lens capsule*

Lens capsules from (*a*) 1-month-old calf and (*b*) 12-month-old steer were reduced as described in the text. The amino acids in acid hydrolysates were separated with a Technicon AutoAnalyzer by using formic acid/pyridine buffers. Peak 1, hexosyl-hydroxylysine; peak 2, hexosyl-lysines; peak 3, hexosyl-lysine dehydration product; peak 4, dihydroxylysinonorleucine; peak 5, hydroxylysinonorleucine (not confirmed); peaks 6 and 7, unknowns. The areas indicate the elution positions of ninhydrin-positive amino acids in the hydrolysate of lens capsule.

complex pattern. The major peaks from the adult bovine lens capsules (Fig. 4) were identified as hexosylhydroxylysine and the hexosyl-lysines, and relatively small peaks were obtained in the known elution positions of the reducible cross-links, a pattern typical of mature collagen (Robins *et al.*, 1973). Analysis of 1-month-old calf lens capsules (Fig. 4) revealed a higher proportion of the reducible cross-links, the major reduced cross-link being hydroxylysinonorleucine. The large amount of hexosyl-lysines present indicated rapid maturation of the lens capsule. Foetal lens capsule contained greater amounts of both dihydroxylysinonorleucine and hydroxylsinonorleucine, but the relative proportion varied between samples and insufficient material was available to demonstrate whether this reflected age differences between the samples. The stable hydroxylysino-5-oxonorleucine could also be detected in pepsin-solubilized collagen from 1-month-old calf lens capsules (results not shown).

Role of polymers in assembly of the rat lens capsules

Previous gel-electrophoretic studies (Heathcote *et al.*, 1978) indicated that the basement-membrane

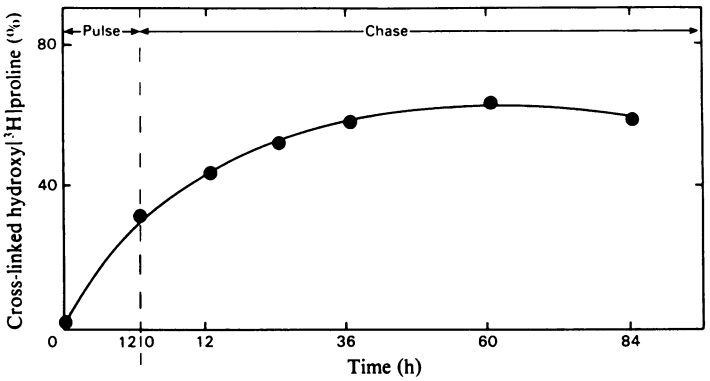


Fig. 5. *Biosynthesis of cross-linked collagenous material by intact rat lenses*
Intact lenses (18) were incubated with [^{3}H]proline (300μCi) in 5ml of DME medium for 12h, and for 12h followed by a chase period of up to 84h in the presence of non-radioactive proline (100μ/ml) and cycloheximide (100μg/ml). After incubation the capsular proteins were analysed as described in the legend to Fig. 1. The proportion of newly synthesized 4-hydroxy[^{3}H]proline present in the void volume of a 4%-agarose column was calculated from the elution profile.

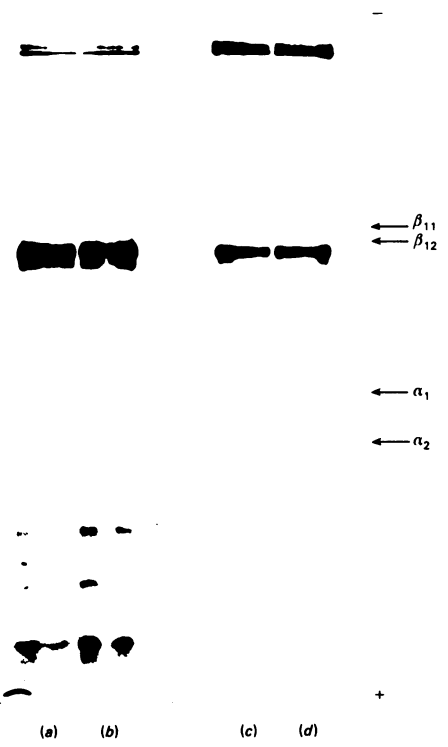


Fig. 6. *Fluorogram of ^{3}H-labelled polypeptides synthesized by intact rat lenses*
Intact lenses (20) were incubated with [^{3}H]proline (500μCi) in 5ml of DME medium for 12h and for 12h followed by a chase period of 60h in the presence of non-radioactive proline (100μg/ml) and cycloheximide (100μg/ml). After incubation proteinase inhibitors were added to the mixture, and the capsules were peeled off the lenses and prepared for electrophoresis as described in the text. Electrophoresis was carried out over approx. 10h at 16mA and the tracking dye was allowed to run off the slab gel. The migration positions of ^{3}H-labelled chick tendon collagen subunits are indicated. Tracks (*a*) and (*b*): 12h pulse-label; tracks (*c*) and (*d*): 12h pulse-label, 60h chase.

collagen precursor is a major component of the lens capsule. It would thus appear that, although a sizeable proportion of the newly synthesized precursor is assembled into cross-linked polymers, this is not the fate of all the precursor; some is integrated within the basement membrane and stabilized by other types of covalent bonds, e.g. disulphide bridges.

To investigate this point further, intact lenses were labelled with [^{3}H]proline for 12h and the incubation was continued in the presence of non-radioactive proline for periods of up to 84h. The ^{3}H-labelled polypeptides were analysed by gel-filtration chromatography, and the proportion of 4-hydroxy-[^{3}H]proline in the void-volume peak was calculated. Fig. 5 shows that after a chase period of 36h the formation of polymer reached a plateau at approx. 60%, suggesting that 40% of the newly synthesized collagen precursor remained un-cross-linked by the hydroxylysine-derived bond.

Electrophoretic analysis of the ^{3}H-labelled polypeptides synthesized by intact lenses indicated that the basement-membrane collagen precursor and high-molecular-weight material, which barely entered the separating gel, were the major radioactive species (Fig. 6). The basement-membrane collagen

precursor was resolved into two bands on the fluorogram of the slab gel, compared with only a single peak of radioactivity in slices of disc gels (Heathcote *et al.*, 1978), and became assembled into the high-molecular-weight aggregates with time. When fluorograms were scanned and the relative proportions of precursor and polymers were determined from the densitometry profile, it was found that after a 12h pulse-label with [^{3}H]proline approx. 15% of the radioactivity was present in the aggregated form. When the pulse-label was followed by a 60h chase period in the presence of non-radioactive proline the proportion of polymeric material increased to about 53%. These values are in reasonable agreement with those obtained by gel-filtration chromatography and hydroxy[^{3}H]proline determination.

Discussion

In previous studies of the biosynthesis of rat lens-capsule collagen, freshly isolated lens capsules were shown to synthesize a collagenous polypeptide of apparent mol.wt. 180000 (Heathcote *et al.*, 1978). With time, a proportion of this polypeptide was assembled into material of higher molecular weight, but, unlike the pro-α-chains of interstitial collagens, none was processed extracellularly to a smaller polypeptide. Although this lack of processing might simply reflect dilution of the converting enzyme in the incubation medium, the demonstration of a 180000-mol.wt. component in the intact lens capsule indicated that conversion of the basement-membrane collagen precursor probably does not occur *in vivo* either. Similarly, the pulse-label and chase studies with intact lenses described in the present paper confirm that conversion of the precursor into a smaller species does not occur and that the formation of high-molecular-weight collagenous material is a characteristic feature of basement-membrane assembly. Furthermore, these experiments suggest that the high-molecular-weight collagenous material is a polymer of the precursor, since each peak in the chromatographic profile (peaks 1 and 2, Figs. 1*b* and 2) has a similar degree of hydroxylation of proline. In analogous experiments with isolated lens capsules the degree of hydroxylation of the high-molecular-weight complex was found to be only half that of the precursor, and this was tentatively interpreted as an indication of the formation of a complex between the precursor and a non-collagenous glycoprotein (Heathcote *et al.*, 1979). This difference may be explained by the limited capacity of the isolated capsules to achieve complete hydroxylation of collagen under the culture conditions used (Heathcote, 1978).

It is not possible to provide a molecular weight for the cross-linked material: since it is eluted in the void volume of the agarose column (Figs. 1 and 2) it may comprise both dimers and higher polymers. However, analysis of the ^{3}H-labelled deoxycholate-insoluble lens-capsule preparation (Fig. 2) emphasizes that both the precursor and its polymers are genuine biosynthetic subunits of the basement membrane. In the experiment described in Fig. 1(*b*) the polymeric material accounts for 45% of the 4-hydroxy[^{3}H]-proline synthesized, whereas in the deoxycholate-treated lens capsule (Fig. 2) the corresponding value is 55%. This may represent a loss of some of the precursor, which is either intracellular or not firmly bound in the basement membrane. At present an explanation of the lack of the coincidence of the maximum radioactivity of proline and hydroxyproline in peak 1 of Fig. 2 has not been found, although it may be related to the presence of two components in the basement-membrane collagen precursor (Fig. 6). The relationship of these two components requires investigation, and it is noteworthy that similar polypeptides are synthesized by cultures of human amniotic-fluid cells (Crouch & Bornstein, 1979).

The occurrence of lysine- and hydroxylysine-derived cross-links in basement membranes was first described by Tanzer & Kefalides (1973), who found a number of putative cross-links in hydrolysates of lens capsules reduced with NaB^3H_4. Identification was based on the elution positions of known cross-links in a single chromatographic system in which the hexosyl-lysines were not resolved from dihydroxylysinonorleucine. The radioactivity in these peaks represented about 5% of the total. T. Sato & R. G. Spiro (unpublished work, cited by Hudson & Spiro, 1972*b*) reported insignificant amounts of reducible cross-links in bovine glomerular basement membrane. Previous studies have shown that the proportion of reducible cross-links in connective tissues decreases with maturation, while the proportion of the hexosyl-lysines increases (Robins *et al.*, 1973). Since the eye matures early in life and the turnover of lens-capsule collagen is low (Rafferty & Goossens, 1978), the proportion of reducible cross-links present in lens capsule from mature animals would be expected to be very small. This suggestion of rapid maturation is supported by our comparison of the reducible cross-links in 1-month-old calf and 12-month-old steer lens capsules (Fig. 4); the cross-links were barely detectable in capsules from the older animals. These findings, and the previous demonstration that β-aminopropionitrile inhibits the formation of high-molecular-weight collagenous material (Heathcote *et al.*, 1978), indicate that the biosynthetic approach offers a more effective means of investigating the initial stages in the cross-linking of basement membranes than does direct chemical analysis.

In these studies it has been possible to identify the reducible cross-link hydroxylysino-5-oxonorleucine in the lens capsule of young rats by using [^{3}H]lysine to label the cross-link specifically (Fig. 3). Hydroxylysino-5-oxonorleucine was the only cross-link detected, and its identity was confirmed in two chromatographic systems and by periodate oxidation of the isolated cross-links. The existence of the cross-link in young bovine lens capsules indicated that biosynthesis *in vitro* was not an artifact of the culture conditions. The physiological significance of the bond is not certain, but it may hold the collagenous molecules in a linear array, thus producing the filamentous substructure displayed by the lens capsule in electron micrographs (Fisher & Wakely, 1976; Heathcote, 1978). It is quite probable that the cross-link occurs in other basement membranes, and there is indirect evidence for its existence in the glomerular basement membrane of young rats (Heathcote *et al.*, 1979). In the rat lens capsule 60% of the newly synthesized basement-membrane collagen precursor is cross-linked by hydroxylysino-5-oxonorleucine, and the percentage in the glomerular basement membrane is probably smaller (Heathcote *et al.*, 1979), a fact that may have implications for both the function and turnover of these membranes.

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