Assembly of chick and bovine lens-capsule collagen

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(Received 14 August 1984/Accepted 9 November 1984)

Chick-embryo and adult bovine lens-capsular epithelia in organ culture synthesized 4-hydroxy[³H]proline-containing polypeptides when incubated in the presence of [³H]proline. These collagenous polypeptides of apparent M_r 180 000, 175 000 and 160 000 became incorporated with time into aggregates of higher molecular size. The formation of such aggregates was inhibited when the tissues were labelled in the presence of β -aminopropionitrile, thereby implicating lysine-derived cross-links in aggregate formation. When the tissues were incubated in the presence of tunicamycin, the collagenous polypeptides synthesized exhibited increased electrophoretic mobilities on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The addition to lens-capsule incubation medium of $\alpha\alpha'$ -bipyridine led to the synthesis of underhydroxylated type IV collagen, also of increased electrophoretic mobility. Extended pulse-chase experiments indicated that such underhydroxylated collagen did not participate in aggregate formation, but was at least as stable as fully hydroxylated non-cross-linked collagen synthesized in the presence of β -aminopropionitrile. Native type IV collagen, recovered from the culture medium when capsules were incubated with [³H]proline for 24 h, was purified by ion-exchange chromatography. Separations conducted on CM-cellulose under denaturing and non-denaturing conditions suggested that the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains occur in the same heterologous triple helix. Densitometric analyses of appropriate fluorograms indicated that these two polypeptides occur in a 2:1 ratio, suggesting that lens-capsule collagen is synthesized as a triple-helical molecule of composition [$\alpha 1(IV)$]₂ $\alpha 2(IV)$.

The lens capsule is of considerable interest because it is the thickest basement membrane in the body and the one most easily isolated. Although evidence suggests that each basement membrane is unique, the lens capsule has been used extensively as a model for morphological, biochemical and biophysical investigations of these important matrices (for review see Heathcote & Grant, 1981). Biosynthetic studies carried out with lenses isolated from 19-day chick embryos (Grant *et al.*, 1972*a,b*, 1973) initially indicated that lens-capsule collagen was synthesized as a precursor that was then converted into a smaller species. In subsequent studies with chick capsules, the conversion could not be demonstrated (Heathcote & Grant, 1982), and studies on the isolated rat lens capsule (Heathcote *et al.*, 1978, 1980) and other basement membranes such as the rat-embryo parietal yolk sac (Minor *et al.*, 1976) and the glomerular basement membrane (Heathcote *et al.*, 1979; Cohen & Surma, 1980) also failed to demonstrate this conversion. In all these later studies proteolytic degradation during the ana-

lytical procedures was minimized by the inclusion of proteinase inhibitors, and this has led to the characterization of a type IV collagen molecule whose constituent polypeptides are of apparent M_r approx. 180 000.

In the present study a comparison of the biosynthetic activities of both chick and bovine lens capsules was undertaken. Partial characterization of the newly synthesized lens-capsule collagen was achieved, and the influence of β -aminopropionitrile, tunicamycin and $\alpha\alpha'$ -bipyridine on the assembly of type IV collagen investigated. Evidence is also presented that suggests that the collagenous polypeptides are secreted in a single heterologous triple-helical molecule of composition [$\alpha 1(IV)$]₂ $\alpha 2(IV)$.

Experimental

Materials

Chick embryos were obtained by incubating fertile eggs purchased from Northern Biological

Supplies, St. Annes, Lancs., U.K., for 19 days at 37°C in a moist atmosphere in a Westernette Egg Incubator (Western Incubators, Chelmsford, Essex, U.K.). Dulbecco's modification of Eagle's medium, foetal bovine serum, penicillin G and streptomycin were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. L-[5-³H]-Proline (26 Ci/mmol) and [³H]acetic anhydride (5 mCi/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Acrylamide, *NN'*-methylenebisacrylamide and $\alpha\alpha'$ -bipyridine were supplied by BDH Chemicals, Poole, Dorset, U.K., and phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, cycloheximide, dithiothreitol, β -aminopropionitrile, tunicamycin, Trizma base and pepsin were purchased from the Sigma Chemical Co., Poole, Dorset, U.K. CM-cellulose (CM52) and DEAE-cellulose (DE52) were obtained from Whatman Chemical Separations, Maidstone, Kent, U.K. 2,5-Diphenyloxazole and chymotrypsin were obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Purified bacterial collagenase was supplied by Advanced Biofactures, New York, NY, U.S.A., and Dulbecco's A phosphate-buffered saline was purchased from Oxoid, Basingstoke, Hants., U.K. Guinea-pig anti-(bovine type IV collagen) was a gift from Dr. S. Ayad of this department. Rat tail-tendon collagen was prepared by the method of Steven & Jackson (1967) and was radiolabelled with [³H]acetic anhydride (Gisslow & McBride, 1975).

Isolation and incubation of chick lenses and bovine lens capsules

Lenses were isolated from chick embryos as previously described (Heathcote & Grant, 1982) and either intact lenses or capsules with their attached epithelia were used. Eyes from freshly killed adult cattle were dissected and the anterior capsules together with their adherent epithelia were isolated. Capsules and lenses were maintained in modified Krebs medium (Dehm & Prockop, 1971) at 4°C before incubation.

Intact chick lenses or chick lens capsules were preincubated for 15 min at 37°C in 3 ml of Dulbecco's modification of Eagle's medium containing 10% (v/v) dialysed foetal bovine serum plus penicillin G (100 i.u./ml) and streptomycin (100 µg/ml). [5-³H]Proline (50 µCi/ml) was then added and the incubation was allowed to continue for a further 24 h at 37°C in a moist atmosphere of CO₂/air (1:19). Similar conditions were employed for the incubation of samples of adult bovine lens capsule, except that a preincubation period of 90 min was found to provide optimum conditions for [³H]proline incorporation. Each bovine capsule was divided into four segments, and segments from

a number of capsules were pooled to minimize potential biological variation. In pulse-chase studies the labelling period with [³H]proline was for 6 h, after which time the incubation was either stopped or allowed to continue for a further 24 h in the presence of non-radioactive proline (100 µg/ml) and cycloheximide (100 µg/ml) (Uitto, 1977). Incubations were terminated by the addition of $\alpha\alpha'$ -bipyridine (1 mM), cycloheximide (100 µg/ml), phenylmethanesulphonyl fluoride (2 mM), *N*-ethylmaleimide (10 mM) and EDTA (25 mM) to the final concentrations indicated. In experiments in which intact chick lenses had been incubated, the samples plus proteinase inhibitors were kept for 15 min at room temperature before the lens capsules were peeled off the bodies of the lenses under a dissecting microscope and sonicated (3 × 30 s) in ice-cold Dulbecco's A phosphate-buffered saline containing phenylmethanesulphonyl fluoride (2 mM), *N*-ethylmaleimide (10 mM) and EDTA (25 mM) with a MSE Soniprep 150 sonicator (22 µm peak to peak). Bovine lens capsules were similarly treated after incubation with [³H]proline.

The effect of β -aminopropionitrile (50 µg/ml), tunicamycin (10 µg/ml) and $\alpha\alpha'$ -bipyridine (10 µg/ml) on collagen biosynthesis in the chick lens was also investigated. These experiments were conducted by supplementing incubation media with these reagents throughout the incubation period and for a 1 h preincubation period.

Enzymic digestion procedures

Bacterial collagenase. Samples of sonicated [³H]proline-labelled lens capsules were suspended in 50 mM-Tris/HCl buffer, pH 7.4 (2 ml), containing 5 mM-CaCl₂, 2 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride and were dialysed against two changes of this buffer (2 × 50 ml, 2 h, 4°C). The tissue suspensions were divided into two equal portions, to one of which was added bacterial collagenase (approx. 200 units/ml), the other being treated with an equal volume of buffer. Both portions were incubated for 6 h at 37°C while dialysing against two changes of buffer. The samples were then dialysed against 0.5 M-acetic acid containing 25 mM-EDTA (6 × 500 ml, 18 h, 4°C) before being freeze-dried.

Pepsin. Radiolabelled lens capsules were suspended in 0.5 M-acetic acid (1 ml) containing pepsin (100 µg/ml) and incubated for 18 h at 4°C. Digests were dialysed against 0.5 M-acetic acid (6 × 500 ml, 18 h, 4°C) and freeze-dried before being subjected to electrophoretic analysis.

Chymotrypsin. Radiolabelled lens capsules suspended in 50 mM-Tris/HCl buffer, pH 7.4 (1 ml), containing 0.4 M-NaCl and chymotrypsin (200 µg/ml) were incubated for 2 h at room tem-

perature. The samples were then dialysed against 0.5M-acetic acid (6 × 500ml, 18 h, 4°C) and freeze-dried before being subjected to electrophoretic analysis.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Analysis of ^3H -labelled macromolecules was effected by using the discontinuous electrophoretic system of Laemmli (1970), and radiolabelled species were detected by fluorography (Heathcote & Grant, 1982).

The 4-hydroxy[^3H]proline contents of individual bands identified by fluorography were determined. [^3H]Proline-labelled polypeptides in the gel were located by overlaying the developed fluorogram on the gel and then carefully excised. The individual bands were hydrolysed in 6M-HCl at 110°C for 18 h and analysed by using the method of Juva & Prockop (1966).

DEAE-cellulose chromatography

Samples were incubated at 100°C for 5 min in 25mM-Tris/HCl buffer, pH 8.6 (2.0ml), containing 4M-urea and 50mM-dithiothreitol. After 2 h incubation at 37°C, the suspension was made 100mM with respect to iodoacetamide and its pH was adjusted to 8. After a 30 min incubation at room temperature, the sample was dialysed against the above buffer for 2 h (2 × 250ml) before centrifugation at 24000g for 10 min. The supernatant was applied to a short column (3cm × 1.5cm) of DE52 DEAE-cellulose equilibrated with 25mM-Tris/HCl buffer, pH 8.6, containing 4M-urea (starting buffer). Elution was performed at 45°C at a flow rate of 70ml/h, with a linear salt gradient provided by 200ml of starting buffer and 200ml of limit buffer (starting buffer containing 0.15M-NaCl) in the respective chambers of a constant-level device.

Samples for analysis under non-denaturing conditions were dialysed against 25mM-Tris/HCl, pH 8.6 (4 × 250ml, 4 h, 4°C), before analysis. Elution was performed as described above, except that elution was at 4°C in buffer containing only 1M-urea.

CM-cellulose chromatography

Samples were prepared for analysis under denaturing conditions by incubation at 100°C for 5 min in 50mM-Tris/HCl buffer, pH 8.6, containing 4M-urea and 50mM-dithiothreitol. After a 2 h incubation at 37°C, the sample was made 100mM with respect to iodoacetamide and its pH was adjusted to 8. After an incubation at room temperature for 30 min, the sample was dialysed against four changes of 50mM-sodium acetate buffer, pH 5.0 (4 × 250ml), before centrifugation at

24000g for 10 min. The supernatant was applied to a column (3cm × 1.5cm) of CM52 CM-cellulose equilibrated with 50mM-acetate buffer, pH 5.0, containing 4M-urea (starting buffer) and chromatography was performed at 45°C at a flow rate of 70ml/h, with a linear gradient of 200ml of starting buffer and 200ml of limit buffer (starting buffer containing 0.15M-NaCl) in the respective chambers of a constant level device.

Samples for analysis under non-denaturing conditions were dialysed against 50mM-acetate buffer, pH 5.0 (4 × 250ml, 4 h, 4°C), containing 1M-urea before analysis. Elution was performed as described above, except that the column and buffers were maintained at 4°C and all solutions contained 1M-urea.

Results

Incorporation of [^3H]proline into collagenous polypeptides

When intact chick-embryo lenses were incubated for 24 h in medium supplemented with [^3H]proline, 4-hydroxy[^3H]proline was found to account for only 3% of the ^3H incorporated into non-diffusible lens macromolecules. In these experiments little, if any, non-diffusible radiolabel was present in the incubation medium. In similar experiments in which isolated chick-embryo lens capsules with their epithelia attached were incubated with [^3H]proline, up to 8% of the incorporated radiolabel was in the form of 4-hydroxy[^3H]proline. The proportion of peptide-bound [^3H]proline found in the capsular incubation medium varied from 14 to 23%, and the hydroxy[^3H]proline content of the medium macromolecules was comparable with that of the whole incubation, suggesting that no preferential release of collagenous proteins into the medium occurred under the conditions used. Supplementation of the incubation medium with ascorbic acid (50 µg/ml) had no effect on the incorporation of [^3H]proline or on 4-hydroxy[^3H]proline synthesis.

Similar studies were conducted with isolated adult bovine anterior lens capsules incubated for up to 24 h with [^3H]proline. The formation of 4-hydroxy[^3H]proline accounted for 6–8% of the total radioactivity incorporated into non-diffusible macromolecules. When the medium was assayed separately it was found that, after 24 h, approx. 40% of the total ^3H incorporated was associated with medium macromolecules and 14% of this non-diffusible label was present as 4-hydroxy[^3H]proline. It was possible to precipitate these collagenous peptides from the culture medium by addition of $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation to yield a precipitate in which 4-hydroxy[^3H]proline represented approx. 23% of the total ^3H in this fraction.

Electrophoretic analysis of newly synthesized [^3H]proline-labelled polypeptides

The nature of the major species synthesized by chick and bovine lens capsules incubated with [^3H]proline was investigated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis followed by fluorography. Two major high- M_r [^3H]proline-labelled polypeptides of M_r 180 000 and 160 000 were detected in extracts of the chick (Fig. 1a) and bovine lens capsules (Fig. 2). The bands of M_r 180 000 and 160 000 from the chick lens capsules had approx. 47% and 44% respectively of their total radioactivity present as 4-hydroxy[^3H]proline. Similar analyses conducted on the two bands recovered from gels of bovine lens capsular material indicated that approx. 40% of the incorporated ^3H was present as 4-hydroxy[^3H]proline in both polypeptides. In both instances the very-high- M_r polypeptides barely entering the gel contained 32–34% of their ^3H as 4-hydroxy[^3H]proline.

In some experiments (see Fig. 3) the species of M_r 180 000 was resolved into a closely spaced doublet whose components were calculated to be of apparent M_r 180 000 and 175 000 (based on collagenous standards of known M_r). It is particularly noteworthy that this doublet represented the only high- M_r collagenous components in culture medium of chick and bovine capsules, and the species of M_r 160 000 was found only in association with capsular material. Preliminary experiments indicated the species of M_r 180 000 and 160 000 to have similar fragmentation patterns when digested with *S. aureus* V8 proteinase (results not shown). When the macromolecules precipitated at 30% $(\text{NH}_4)_2\text{SO}_4$ saturation from the culture medium in which bovine capsules had been incubated with [^3H]proline for 24 h were redissolved in phosphate-buffered saline and incubated with anti-(bovine type IV collagen) serum, the only component immunoprecipitated electrophoresed as a broad band with apparent M_r 180 000 (Fig. 2). This band was shown to contain 49% of its radiolabel present in the form of 4-hydroxy[^3H]proline.

Influence of bacterial collagenase, pepsin and chymotrypsin on the newly synthesized collagenous polypeptides

The collagenous nature of the major high- M_r [^3H]proline-labelled polypeptides synthesized by chick-embryo and adult bovine lens capsules was confirmed by their complete susceptibilities to highly purified bacterial collagenase (Figs. 1b and 2). In contrast, these polypeptides exhibited resistance to digestion by pepsin and by chymotrypsin when the [^3H]proline-labelled capsules were incubated under conditions in which the triple-helical

Fig. 1. Enzymic digestion of radiolabelled chick lens capsules

Four groups of 20 chick lenses were incubated with [^3H]proline (75 μCi) for 24 h in Dulbecco's modification of Eagle's medium (1.5 ml) containing 10% (v/v) dialysed foetal bovine serum. The incubation was stopped and proteinase inhibitors were added as described in the text, except that EDTA was omitted. The lens capsules were isolated and incubated with bacterial collagenase, pepsin and chymotrypsin as described in the Experimental section. Equal amounts of each sample were subjected to electrophoretic analysis, and radiolabelled species were detected by fluorography. Fluorograms were then scanned with an LKB laser densitometer. Trace (a) represents the control sample after incubation for 6 h at 37°C in 50 mM-Tris/HCl buffer, pH 7.4. Trace (b) represents a sample after incubation with bacterial collagenase, and traces (c) and (d) represent samples incubated in the presence of pepsin and chymotrypsin respectively. The electrophoretic mobilities of type I collagen β_{11} (1), β_{12} (2), α_1 (3) and α_2 (4) chains are indicated, and the position of the dye front is shown by the heavy arrow.

Fig. 2. Identification of newly synthesized bovine lens-capsule collagen

Bovine lens capsules (four) were incubated with [^3H]proline (50 $\mu\text{Ci/ml}$) for 24 h as described in the text. The lens capsules were isolated, sonicated, and collagenous polypeptides were identified by their susceptibility to bacterial collagenase. Digestion was for 6 h, and samples were then subjected to electrophoretic and fluorographic analyses. Collagenous polypeptides secreted into the culture medium were immunoprecipitated with anti-(type IV collagen) serum. Samples in tracks 1–4 are as follows: 1, control capsular proteins; 2, bacterial-collagenase digest of capsular proteins; 3, non-immunoprecipitated medium proteins; 4, immunoprecipitate of medium collagenous polypeptides. The mobilities of type I collagen β_{11} (1), β_{12} (2), α_1 (3) and α_2 (4) chains are indicated, and the positions of the dye fronts are shown by the heavy arrows.

Fig. 3. Electrophoretic analysis of chick lens-capsule macromolecules synthesized in the presence of β -aminopropionitrile, tunicamycin and $\alpha\alpha'$ -bipyridine

Incubations were carried out in medium (3 ml) supplemented with β -aminopropionitrile (50 $\mu\text{g/ml}$), tunicamycin (10 $\mu\text{g/ml}$) or $\alpha\alpha'$ -bipyridine (10 $\mu\text{g/ml}$). Duplicate groups of chick lenses (2×40) were incubated for 6 h with [^3H]proline (50 $\mu\text{Ci/ml}$). One incubation was then stopped, whereas the other was allowed to continue for a further 24 h in the presence of unlabelled proline (100 $\mu\text{g/ml}$) and cycloheximide (100 $\mu\text{g/ml}$). After the incubation, proteinase inhibitors were added, and the capsules were isolated by dissection and capsular extracts were analysed on 5%-polyacrylamide gels as described in the text. Samples of tracks 1–7 are as follows: 1, control incubation (6 h); 2, incubation (6 h) in the presence of tunicamycin; 3, incubation (6 h) followed by a 24 h chase with tunicamycin present throughout; 4, incubation (6 h) in the presence of β -aminopropionitrile; 5, incubation (6 h) followed by a 24 h chase with β -aminopropionitrile present throughout; 6, incubation (6 h) in the presence of $\alpha\alpha'$ -bipyridine; 7, incubation (6 h) followed by 24 h chase with $\alpha\alpha'$ -bipyridine present throughout. The mobilities of type I collagen β_{11} (1), β_{12} (2), α_1 (3) and α_2 (4) chains are indicated, and the position of the tracker dye is shown by the heavy arrow.

collagen type IV remained native (Figs. 1c and 1d). Peptic digestion gave rise to a 4-hydroxy[^3H]proline-rich fraction containing two major bands. These polypeptides, of approx. M_r 110000 and 95000, are presumed to correspond to $\alpha_1(\text{IV})$ and $\alpha_2(\text{IV})$ species, initially designated as the C and D

chains of lens-capsule collagen (Gay & Miller, 1979). Chymotryptic digestion generated two 4-hydroxy[^3H]proline-containing bands of apparent M_r 140000 and 125000 (Fig. 1d).

Influence of β -aminopropionitrile, $\alpha\alpha'$ -bipyridine and tunicamycin on the synthesis of [^3H]proline-labelled polypeptides

The occurrence of very-high- M_r collagenous polypeptides only just entering the 5%-polyacrylamide gels run under reducing conditions was noted in the short-term incubations. The relative quantity of this high- M_r material in the chick lens capsule could be increased from approx. 14% to approx. 32% if the 6 h labelling period was followed by a 24 h chase period in the presence of unlabelled proline and cycloheximide. Virtually identical results were obtained with the bovine lens capsules incubated under these conditions, but in this instance it was found that approx. 18% of the 4-hydroxy[^3H]proline was in very-high- M_r material after a 6 h incubation and that this value rose to 30% after a 24 h chase period (results not shown). However, when these pulse and pulse-chase experiments were conducted in the presence of β -aminopropionitrile, the proportion of radioactivity incorporated into the high- M_r material was substantially diminished, suggesting that these aggregates were stabilized by lysine- and/or hydroxy-lysine-derived cross-links. As expected, the collagenous polypeptides synthesized in the presence of β -aminopropionitrile were of similar M_r to those produced in the control incubation and had similar degrees of proline hydroxylation (Table 1). The failure to detect any lower- M_r collagenous components during the pulse-chase experiments confirmed previous studies on the rat lens capsule (Heathcote *et al.*, 1980), indicating that the newly

synthesized collagenous polypeptides are incorporated directly into higher- M_r aggregates.

When chick-embryo lens capsules were incubated in the presence of tunicamycin, collagenous polypeptides having a normal degree of proline hydroxylation (Table 1) were synthesized, but their electrophoretic mobilities had increased (Fig. 3). The mobility of a species, M_r 120000, detectable in the incubation was markedly affected by tunicamycin, indicating that it probably had a significant carbohydrate content. The nature of this component was not investigated further, but staining of the gels with periodic acid/Schiff reagent confirmed the presence of a glycoprotein of this size in the solubilized extract of chick-embryo lens capsule.

The addition of $\alpha\alpha'$ -bipyridine to the incubations of chick lens capsules also had a marked effect on the electrophoretic mobilities of the major high- M_r [^3H]proline-labelled species (Fig. 3). Two bands of approx. M_r 150000 and 140000 were synthesized under these conditions, and the absence of 4-hydroxy[^3H]proline from these components (Table 1) was consistent with their being the unhydroxylated precursors of type IV collagen. The glycoprotein of M_r 120000 produced in the presence of β -aminopropionitrile had the same mobility when synthesized in the presence of $\alpha\alpha'$ -bipyridine, indicating it to be non-collagenous.

When a 6 h incubation of chick lens capsules in the presence of $\alpha\alpha'$ -bipyridine was followed by a 24 h chase, it was observed that the synthesis of high- M_r aggregates did not occur (Fig. 3). Also noteworthy was the fact that no significant

Table 1. *Species synthesized by chick lens capsules under a variety of incubation conditions*
Selected radiolabelled species shown in Fig. 3 were isolated as described in the text and assayed for 4-hydroxy[^3H]proline.

Incubation conditions	$10^3 \times$ Relative M_r of species produced	$\frac{\text{d.p.m. in Hyp}}{\text{d.p.m. in Pro + Hyp}} \times 100$ (%)
Control	180	41
	175	44
	160	45
	120	0
Tunicamycin (10 $\mu\text{g/ml}$)	165	53
	160	47
	150	43
	110	0
β -Aminopropionitrile (50 $\mu\text{g/ml}$)	180	37
	175	45
	160	42
	120	0
$\alpha\alpha'$ -Bipyridine (10 $\mu\text{g/ml}$)	150	2
	140	1
	120	0

degradation of the unhydroxylated procollagen polypeptides occurred; and, in further experiments, when the chase period was extended to 72 h, the unhydroxylated procollagen was of comparable stability with the fully hydroxylated collagen synthesized in the presence of β -aminopropionitrile (results not shown).

Analysis of newly synthesized macromolecules by ion-exchange chromatography

When extracts of radiolabelled chick lens capsules were subjected to DE52 DEAE-cellulose chromatography under denaturing conditions, the newly synthesized collagenous components did not bind (Fig. 4). This pre-gradient fraction (fraction A, Fig. 4) contained over 90% of the 4-hydroxy-[3 H]proline recovered from the column, and when analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was found to contain three major polypeptides of apparent M_r 180 000, 175 000 and 160 000. Almost identical results were obtained in studies conducted with bovine lens-capsular extracts labelled with [3 H]proline (results not shown). For the chick lens-capsule extracts it was noteworthy that two major [3 H]proline-labelled non-collagenous polypeptides of apparent M_r 170 000 and 120 000 (based on collagenous standards of known M_r) were present in the material eluted in the gradient in fractions B–D and fraction I (Fig. 4) respectively.

Further characterization of the arrangement of the subunits in the lens-capsule collagen was hampered by the difficulty of extracting native protein from intact tissue as a result of its high degree of cross-linking by disulphide bonds and lysine-derived bonds. However, substantial amounts of triple-helical lens-capsule collagen were secreted by isolated capsules into the culture medium and provided a source of native protein. The failure of type IV collagen to bind to DEAE-cellulose was exploited as a simple purification step. Thus [3 H]proline-labelled medium macromolecules were separated on DE52 DEAE-cellulose at 4°C under non-denaturing conditions, and the collagenous polypeptides eluted in the unbound fraction were then analysed on CM-cellulose under both non-denaturing and denaturing conditions. The native collagen was eluted as a single 4-hydroxy[3 H]proline-containing peak (peak A, Fig. 5a) at a salt concentration of 0.12 M when chromatographed on CM-cellulose under non-denaturing conditions. When the sample was reduced, alkylated and denatured before analysis, two hydroxy-[3 H]proline-containing peaks (peaks B and C, Fig. 5b) eluted at salt concentrations of 0.08 M and 0.13 M were obtained. Identical results were obtained with the collagenous components released into the culture medium by bovine lens capsules. Electrophoretic analyses of the CM52 CM-cellulose fractions from bovine and chick samples indicated

Fig. 4. Ion-exchange chromatography of [3 H]proline-labelled polypeptides extracted from chick lens capsules. Urea/dithiothreitol extracts of chick lens capsules were subjected to DEAE-cellulose ion-exchange chromatography carried out under denaturing conditions. (a) Elution profile of radioactivity. The arrow indicates the point at which a gradient of 0–0.15 M NaCl was applied to elute polypeptides bound to the column. (b) Electrophoretic and fluorographic analysis of (A) material recovered in the pregradient fraction, and (B–I) material recovered in the gradient fractions indicated. The mobilities of type I collagen β_{11} (1), β_{12} (2), α_1 (3) and α_2 (4) chains are indicated, and the dye front is shown by the heavy arrow.

Fig. 5. Ion-exchange chromatography of [^3H]proline-labelled polypeptides, present in chick lens-capsule incubation medium. Newly synthesized [^3H]proline-labelled collagenous polypeptides were isolated from chick lens-capsule incubation medium by DEAE-cellulose chromatography carried out under non-denaturing conditions. The material so isolated was analysed by CM-cellulose chromatography under (a) non-denaturing and (b) denaturing conditions as described in the text. The arrows indicate the position at which gradients of 0–0.15M-NaCl were applied to elute polypeptides bound to the columns. (c) Electrophoretic and fluorographic analysis of material bound to the columns. Track 1 shows fraction A eluted at a salt concentration of 0.12M from the column of CM52 CM-cellulose run under non-denaturing conditions. Tracks 2 and 3 show fractions B and C eluted from the column run under denaturing conditions at salt concentrations of 0.08M and 0.13M respectively. The mobilities of type I collagen β_{11} (1), β_{12} (2), α_1 (3) and α_2 (4) chains are indicated, and the dye front is shown by the heavy arrow.

that peaks B and C contained polypeptides of M_r 180000 and 175000 respectively. The single hydroxyl[^3H]proline-containing peak (peak A, Fig. 5a) obtained from samples analysed under non-denaturing conditions was found to contain a broad band of approx. M_r 180000 containing the type IV collagen doublet of $\alpha_1(\text{IV})$ and $\alpha_2(\text{IV})$ chains.

It is also noteworthy that, in experiments where the native medium collagens were reduced and alkylated before being chromatographed on CM-cellulose under non-denaturing conditions, a single hydroxyl[^3H]proline-containing peak eluted at 0.12M-NaCl was again obtained (results not shown). These studies clearly demonstrate that the collagenous polypeptides of the lens capsule exist in a single heterologous helix. When those fluorograms, in which clear resolution of the $\alpha_1(\text{IV})$ and $\alpha_2(\text{IV})$ chains was obtained, were analysed by laser densitometry, the two bands were shown to be present in a ratio of approx. $1.83 \pm 0.43:1.0$ (S.D. for 12 fluorograms).

Discussion

The occurrence of a genetically distinct collagen in basement membranes, first proposed by Kefa-

lides (1973), has been clearly established (for review see Heathcote & Grant, 1981). Much information on the structure and properties of type IV collagen has since derived from studies on systems such as the EHS sarcoma (Timpl *et al.*, 1979, 1981; Weber *et al.*, 1984; Yurchenco & Furthmayr, 1984) and cell cultures (Alitalo *et al.*, 1980a,b; Fessler & Fessler, 1982; Duncan *et al.*, 1983) that synthesize considerable amounts of basement membrane components. Details of basement-membrane assembly are still ill-defined, but the isolated lens capsule remains an excellent model system for the study of the synthesis of an authentic basement membrane.

Extraction of [^3H]proline-labelled chick and bovine lens capsules under denaturing and reducing conditions permitted the isolation of fractions rich in collagenous components of apparent M_r 180000, 175000 and 160000. It was noteworthy that the collagenous species of M_r 160000 was not detected in culture medium, but was present only in extracts of the basement-membrane matrix. The relationship of this component to the high- M_r basement-membrane collagen precursors is unclear, but preliminary peptide-mapping studies indicate it to be closely related to the species of M_r 180000. The possibility that the species of M_r

160000 is the result of minor processing or represents a partial degradation artifact would be consistent with other reports that have indicated that acetic acid extracts of rat parietal yolk sac and EHS sarcoma contain type IV collagen polypeptides of M_r 160000 and 140000 (Tryggvason *et al.*, 1980; Kleinman *et al.*, 1982). It is perhaps relevant that in acetic acid extracts of bovine lens capsule species of M_r 160000 and 140000 also predominate (Veis & Schwartz, 1981).

Although no evidence was obtained for the processing of the newly synthesized lens-capsule collagen in a manner analogous to the cleavage that precedes the formation of fibrillar interstitial collagens (for review see Heathcote & Grant, 1980), it was clear from the pulse-chase experiments in the presence and in the absence of β -aminopropionitrile that aggregate formation of the collagenous polypeptides normally occurs with an involvement of lysine- and/or hydroxylysine-derived cross-links. The location of these covalent cross-links in terminal regions of the collagen polypeptides is inferred by recovery of high- M_r collagenous polypeptides from pepsin digests of anterior lens capsule (Schwartz & Veis, 1979a,b) and studies on type IV collagen from placenta (Bailey *et al.*, 1984) provide direct evidence for hydroxylysine-derived keto-imine cross-links at both the *N*-terminal and *C*-terminal regions of type IV collagen.

These terminal regions are believed to play a major role both in assembly of the collagen triple helix and in the extracellular aggregation of the individual type IV collagen molecules to form the insoluble basement-membrane matrix (Timpl *et al.*, 1981; Bailey *et al.*, 1984; Weber *et al.*, 1984; Yurchenco & Furthmayr, 1984). For the interstitial collagens, the terminal propeptides are also the sites of attachment of *N*-linked oligosaccharide side chains (Olsen *et al.*, 1977; Clark & Kefalides, 1978), and evidence for the occurrence of analogous sugar units on the type IV collagen is indicated by the experiments with lens capsules incubated in the presence of tunicamycin. The increased electrophoretic mobility of the collagenous polypeptides synthesized under these conditions (Fig. 3) indicated that type IV collagen is normally glycosylated to a significant extent, an observation consistent with carbohydrate analyses reported for the bovine anterior lens capsule (Fukushi & Spiro, 1969; Kefalides, 1973). Tunicamycin was also observed to influence the electrophoretic mobility of a polypeptide of apparent M_r 120000 (based on the mobilities of collagenous standards of known M_r) which corresponded to a non-collagenous glycoprotein detectable in extracts of chick-embryo lens capsule. Additional glucosyl-galactosyl sugar units linked *O*-glycosid-

ically to hydroxylysine are a characteristic feature of basement-membrane collagens, where they account for up to 10% (w/w) (Fukushi & Spiro, 1969; Kefalides, 1973). Thus, when hydroxylation of proline and lysine residues was inhibited by $\alpha\alpha'$ -bipyridine, the increased electrophoretic mobilities of the type IV precursors can be attributed to their lower M_r values as a consequence of their unhydroxylated and non-glycosylated nature. Lack of hydroxylysine residues would also contribute to the failure of the non-hydroxylated collagen to form any high- M_r aggregates, but lack of hydroxyproline residues would be expected to give rise to collagenous polypeptides incapable of forming a stable triple helix under the physiological conditions used. It was therefore surprising that, in the extended pulse-chase experiments conducted in the presence of $\alpha\alpha'$ -bipyridine, the unhydroxylated components were not degraded, but exhibited a stability comparable with fully hydroxylated type IV collagen. Similar studies on the rat parietal yolk sac indicated that unhydroxylated type IV collagen was capable of being secreted and was surprisingly stable without being incorporated into the extracellular matrix (Maragoudakis *et al.*, 1978).

When intact chick lenses were incubated with [3 H]proline, the newly synthesized collagenous components became incorporated into the capsular material and were not readily solubilized without resort to reduction and denaturation. However, the isolated capsules with attached epithelia secreted significant amounts of type IV collagen monomers into the culture medium, and thereby permitted a study of the chain composition of the native molecules. By using DEAE-cellulose chromatography under non-denaturing conditions it was possible to purify the basement-membrane collagen, and further chromatography on CM-cellulose under both native and denaturing conditions indicated that the collagen consisted of a single heterologous triple-helical molecule. This observation, taken together with the laser-densitometric analyses, which indicated that the polypeptides of M_r 180000 and 175000 occur in a ratio of approx. 2:1, suggest that the lens-capsule collagen is synthesized as a triple-helical molecule of the form $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$, where the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ species are of M_r 180000 and 175000 respectively. Although the existence of homopolymers, $[\alpha 1(\text{IV})]_3$ and $[\alpha 2(\text{IV})]_3$, of the two basement-membrane collagen chains has been considered (Tryggvason *et al.*, 1980; Alitalo *et al.*, 1980b; Robey & Martin, 1981), the existence of a heteropolymer subunit in lens-capsule collagen is consistent with other studies on pepsin-derived type IV collagen preparations derived from chicken gizzard (Mayne & Zettergren, 1980) and kidney cortex (Trueb *et al.*, 1982). Further studies will be required to establish

the true organization of the collagen molecules in the lens capsule, and, although amino acid analyses suggest that the type IV collagen precursors might account for over 90% of the capsule, their interactions with extracellular glycoproteins identified in this study, and with heparan sulphate (Parthasarathy & Spiro, 1982), will need to be considered.

The financial support of the British Diabetic Association is gratefully acknowledged.

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