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Cross-linking in type IV collagen

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Type IV collagen could not be extracted from human placenta using 6M-urea containing 10mM-dithiothreitol, indicating that the type IV molecule is stabilized within the basement membrane by covalent cross-links. However, various forms of type IV collagen molecule were extractable by means of increasingly severe proteolytic conditions. Type IV collagen tetramers ('spiders') lacking only the *C*-terminal globular region (NC1) were further digested to the 'long-form' 7S fragment and an assortment of helical rod-like molecules derived from the 'leg' region. These molecules were separated by salt fractionation and examined by rotary-shadowing electron microscopy. Isolation of these fractions from placenta which had been reduced with NaB^3H_4 revealed that both the 7S (*N*-terminal) and *C*-terminal regions contained significant proportions of reducible lysine-derived cross-links. These cross-links were shown to be exclusively the stable oxo-imine hydroxylysino-5-oxonorleucine. The number of the cross-links per molecule was significantly lower than might be expected in order to fully stabilize the molecule. These results suggest that the keto-imine cross-links in type IV collagen have been stabilized in part by conversion into an unknown non-reducible form, although a sensitive immunoassay failed to show the presence of any pyridinoline. Comparison with the fibrous collagen from placenta suggested that the rate of this conversion is much greater in basement-membrane collagen.

Since the early 1970s considerable interest in the field of connective-tissue research has centred on the structure of basement membranes. In particular, the discovery, by Kefalides and his co-workers [see the review by Bornstein & Sage (1980)], of a novel collagen in basement membranes has led to the involvement of many groups in the purification and characterization of this important protein. Basement membrane or type IV collagen has been isolated from such diverse sources as glomerular basement membrane, lens capsule, placental basement membrane and mouse EHS sarcoma (Bornstein & Sage, 1980). In every case it has been shown to be composed of a complex mixture of protein chains. Early work demonstrated that type IV collagen was composed of two separate chains, which were probably derived from different molecules (Bailey *et al.*, 1979; Glanville *et al.*, 1979; Bornstein & Sage, 1980). An additional protein, later shown to be derived from type IV, could be prepared by extended pepsin solubilization of the tissue. This component was designated '7S collagen' from its characteristics in the analytical ultracentrifuge.

Because so many different fragments of type IV collagen could apparently be produced from a variety of basement membranes merely by varying the severity of pepsin digestion (for reviews, see Bornstein & Sage, 1980; Duance & Bailey, 1983), one or two groups have centred their research efforts to resolve these problems by using the EHS-sarcoma type IV collagen. Through this concerted effort it has been possible to probe more thoroughly the structure of the molecule, and a widely accepted model for type IV collagen structure has been proposed (Timpl *et al.*, 1981).

In this model, based on elegant rotary-shadowing-electron-microscopic results, four type IV molecules are thought to be aggregated into a 'spider-like' tetramer through specific associations of the *N*-terminal regions of each molecule (see Fig. 1*a*) forming the 7s domain. At the *C*-terminal end

Abbreviations used: SDS, sodium dodecyl sulphate; EHS, Engelbreth–Holm–Schwarm; phosphate-buffered saline, 0.15M-NaCl/0.02mM-sodium phosphate buffer, pH7.4; Hyl (in sequences), hydroxylysine.

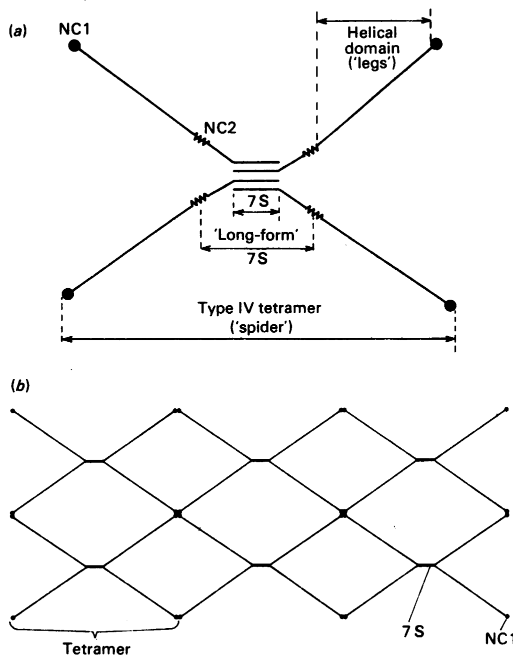

Fig. 1. *Schematic representation of (a) the structural units of type IV collagen ('spider') as proposed by Kuhn et al. (1981) and (b) the 'chicken-wire' lattice of 'spiders' proposed by Timpl et al. (1981)*

of each triple helical 'leg' of the tetramer is a globular domain (termed NCl), which is thought also to undergo reaction with other molecules, thereby polymerizing the type IV tetramers into a specific matrix. Dureau *et al.* (1983) have monitored the biosynthesis of type IV collagen and have shown single pro-type IV procollagen molecules aggregating to form dimers and the dimers then aggregating to form tetramers or 'spiders'. Evidence for the stabilization of the 'spiders' through disulphide bonds in the 7S (*N*-terminal) region is strong, as the fragment isolated from lathyritic EHS-sarcoma (examined by rotary-shadowing electron microscopy) has an M_r in excess of 200000 before reduction with mercaptoethanol but, after reduction, migrates on SDS/polyacrylamide gels as a group of low-M_r peptides (Dixit *et al.*, 1981).

The increased solubility of type IV collagen from lathyritic EHS sarcoma indicates the presence of lysine-derived intermolecular cross-links stabilizing the molecules in the matrix. An early report on gross preparations of basement membrane (Tanzer & Kefalides, 1973) demonstrated the presence of lysine-derived collagen cross-links in Descemet's membrane, renal glomerulus and anterior lens capsule. Heathcote *et al.* (1980), in a more detailed study of cross-link biosynthesis in cultured lens capsule, showed the presence of only one major lysine-derived cross-link, hydroxylysino-5-oxonorleucine. Subsequent studies by Le Pape *et al.* (1981) and Wu & Cohen (1982) on whole basement membranes have confirmed these findings. However, basement membrane is a complex of several proteins, and no data have been forthcoming to confirm the nature or location of the cross-links within and between the type IV molecules.

In the present report we show that 'spider-like' structures, equivalent to those seen from EHS sarcoma, can be observed in controlled pepsin extracts of human placental cotyledons. Also, that long-form and short-form 7S structures can be prepared by extended pepsin treatment of these tetramers and that the 7S region contains reducible hydroxylysino-5-oxonorleucine cross-links as well as disulphide bonds. Further, the 'spiders' themselves are also linked by the same cross-link through the NCl region of the triple helical 'legs'.

Materials and methods

Preparation of collagens from NaB^3H_4-reduced and untreated placenta

Cotyledons were removed from human placentae, cut into 1 cm tubes and washed overnight in running tap water. The washed cotyledons were then homogenized and extracted twice with 0.4M-sodium acetate and twice with phosphate-buffered saline. The insoluble material was collected by centrifugation, weighed (approx. 100g) and divided into two portions. One half was dialysed against distilled water and suspended in 0.5M-acetic acid. The other half was reduced at room temperature for 1h with NaB^3H_4 (Amersham International, Amersham, Bucks., U.K.) at a NaB^3H_4/wet-weight-of-tissue ratio of 1:300. After reduction the excess NaB^3H_4 was removed by lowering the pH to 4.0 with acetic acid, followed by dialysis against distilled water (Robins *et al.*, 1973).

The unreduced and NaB^3H_4-reduced samples were suspended in 0.5M-acetic acid and digested (at a wet-weight/enzyme ratio of 100:1) with pepsin (Sigma, Poole, Dorset, U.K.) for 40h at 4°C. Insoluble material was removed by centrifugation and the solubilized collagens were precipitated by addition of 7% (w/v) NaCl. The precipitate was dissolved in 1M-NaCl/0.05M-Tris/HCl, pH7.5, and dialysed against 0.2M-NaCl/0.05M-Tris/HCl, pH7.5. The molarity of this solution was raised to 2.0M with NaCl in order to precipitate types III and IV collagen. The supernatant was dialysed against 0.5M-acetic acid and type I collagen was precipitated by addition of 0.7M-NaCl. Type IV collagen remained in solution. The 2.0M-NaCl precipitate was dissolved in 0.2M-NaCl, 0.05M-Tris/HCl, pH7.5, and dialysed

against 0.02M-NaCl/0.02M-Tris/HCl, pH 7.5, when type III collagen precipitated and type IV collagen was collected in the supernatant.

Preparation of 'long-form 7S' and helical 'leg' fragments from placental type IV collagen

Type IV collagen isolated from unreduced and NaB^3H_4-reduced placenta was subjected to a second pepsin digestion. The type IV collagen was dissolved in 0.5M-acetic acid (2mg/ml) and pepsin added (pepsin/collagen, 1:10, w/w) and digested for 72h at 20°C. After cooling the mixture to 4°C, the pH was raised to 8.0 with saturated Tris solution in order to denature the pepsin. The collagen solution was then dialysed against 0.5M-acetic acid and the small precipitate which formed was discarded. Addition of 7% NaCl produced a further precipitate, which was collected by centrifugation. Both the precipitate and supernatant were dialysed against 0.5M-acetic acid, freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis and rotary-shadowing electron microscopy.

Non-proteolytic extraction of type IV collagen

Placental cotyledons were washed as described previously with sodium acetate and phosphate-buffered saline and extracted with 0.5M-acetic acid for 72h at 4°C. The solubilized material was dialysed against 0.02M-NaCl/0.02M-Tris/HCl, pH 7.4, and the supernatant was shown by rotary-shadowing electron microscopy to contain typical type IV collagen 'spiders'.

A further portion of washed cotyledons was extracted with 6M-urea/10mM-dithiothreitol/0.02M-Tris/HCl, pH 8.1, for 72h at 4°C. Insoluble material was removed by centrifugation and collagenous proteins precipitated by addition of 10% NaCl. Both the precipitated material and supernatant were analysed by SDS/polyacrylamide-gel electrophoresis and rotary-shadowing electron microscopy.

Preparation of chick anterior lens capsules

Lenses were removed from 15-day and 21-day chick embryos. The lenses were suspended in phosphate-buffered saline and adhering material was removed by ultrasonication. Generally, four to five lenses were sonicated for 1 min in 25ml of ice-cold phosphate-buffered saline using an MSE 150W ultrasonic disintegrator fitted with a 3mm exponential probe. The lens capsules were then pierced with a needle and the contents expelled with a further 30s burst of ultrasound. The ruptured capsules were collected, washed in phosphate-buffered saline and reduced with NaB^3H_4 as described above.

SDS/polyacrylamide-gel electrophoresis

Samples were analysed by SDS/polyacrylamide-gel electrophoresis before and after reduction with 2-mercaptoethanol by the method of Sykes & Bailey (1971). Polyacrylamide (5.5%, w/v) slab gels were run in a 0.05M-Tris/borate buffer and stained with Coomassie Brilliant Blue R-250.

Rotary shadowing

Rotary shadowing was carried out by the method of Kuhn *et al.* (1981). Briefly, collagen preparations were dissolved (0.5mg/ml) in 0.025M-acetic acid/50% (v/v) glycerol and sprayed on to freshly cleaved mica sheets. These were then shadowed with platinum at an angle of 9° and then carbon (at 90°) in the evacuated chamber of an Edwards coating unit (model E306A). The carbon replicas were floated on to distilled water, collected on copper grids and examined in a Philips 400 electron microscope.

Cross-link analysis

Collagen types isolated from unreduced placenta as described above were assessed for purity by SDS/polyacrylamide-gel electrophoresis. The freeze-dried pure collagens were re-dissolved in phosphate-buffered saline (2mg/ml) and reduced with (borohydride/collagen, 1:30, w/w) as described above. The reduced collagens were then dialysed against 0.5M-acetic acid, freeze-dried and hydrolysed in 6M-HCl for 24h and the hydrolysates applied to a Zeolite 225 ion-exchange column developed with pyridine/formate by the method of Robins *et al.* (1973). The identity of the radioactive peaks was confirmed by comparison with authentic standards on a Jeol 6AH amino acid analyser.

Pyridinoline was assayed in type IV collagen preparations as follows. Pure pyridinoline standard was prepared by gel filtration on a column (95cm × 2.5cm) of Sephadex G-10 in 0.1M-acetic acid of acid hydrolysates of washed, decalcified bovine bone. The fluorescent material (monitored at 405nm with excitation at 295nm) was pooled and subjected to ion-exchange chromatography on a 29cm × 1cm column adapted for the purpose on the Jeol 6AH amino acid analyser. Citrate buffers lacking detergents were used and pyridinoline was collected and desalted on Sephadex G-10. The identity of the pyridinoline standard was assessed by spectrophotometric criteria (Elsden *et al.*, 1980). Hydrolysed type IV collagen (20mg) was chromatographed on a column (95cm × 2.5cm) of G-10 Sephadex in 0.1M-acetic acid, and the column effluent was monitored for fluorescence at 405nm with excitation at 295nm. No fluorescent pyridinoline peak was observed, but the portion of the effluent corresponding to the elution position of pyridinoline was pooled and further assayed by

standard amino acid analysis on a Jeol 6AH analyser. In addition, an acid hydrolysate of 10mg of pure type IV collagen was also assayed for pyridinoline by a specific immunoassay, kindly performed by Dr. S. P. Robins as described previously (Robins, 1983*a*).

Results

Acid-soluble type IV collagen preparations

Washed human placenta extracted with 0.5M-acetic acid without added proteinase inhibitors yielded a preparation of whole type IV molecules in the tetrameric form. The molecules were examined by electron microscopy after rotary shadowing (Fig. 2). The tetramers were composed of four long helical molecules containing the previously described 7S, helical ('leg') and NCl domains, which were all clearly visible.

An attempt to solubilize similar molecular forms in 6M-urea containing 10mM-dithiothreitol at pH8.1 was not successful, however. Some soluble material with a high M_r (analysed by SDS/polyacrylamide-gel electrophoresis) was obtained, but it did not contain any type IV collagen tetramers or derivatives as assessed by electron microscopy and by hydroxyproline determination.

Pepsin-liberated type IV polypeptides

Digestion of both untreated and NaB^3H_4-reduced washed placental cotyledons with a pepsin/substrate ratio of 1:10 (w/w) for 40h at 4°C produced identical preparations of type IV collagen as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 3). As can be seen, the typical pattern of three major protein bands with M_r 170000, 100000 and 70000 (as previously reported: Bailey *et al.*, 1979) was obtained from both preparations, showing that reduction with NaB^3H_4 before extraction did not modify the cleavage sites of the enzyme. Rotary-shadowing electron microscopy corroborated the gel-electrophoretic evidence and showed the presence of tetrameric 'spiders' of type IV collagen which lacked the NCl region in both preparations.

A second, prolonged, pepsin digest (pepsin/substrate ratio, 1:10, w/w; 20°C; 72h) cleaved the type IV molecules of both untreated and NaB^3H_4-reduced preparations at the NC2 region. The addition of 7% NaCl precipitated the liberated long helical 'legs' of the tetramers (Fig. 5*a* below), whereas the so-called 'long-form' 7S component remained in solution (Fig. 5*b* below).

Analysis of the precipitated 'legs' on SDS/polyacrylamide gels revealed basically the same pattern from both the NaB^3H_4-reduced (Figs. 4*b* and 4*c*) and the untreated (Figs. 4*d* and 4*e*) placenta. Four major bands were present and, after treatment with mercaptoethanol, two further bands were observed, presumably derived from higher-M_r components at the top of the gel. In contrast, the 7S domain from both the reduced (Figs. 4*f* and 4*g*) and untreated (Figs. 4*h* and 4*i*) placenta revealed a single high-M_r component which was unaffected by mercaptoethanol. The 7S domain extracted from lathyritic EHS sarcoma is readily cleaved by

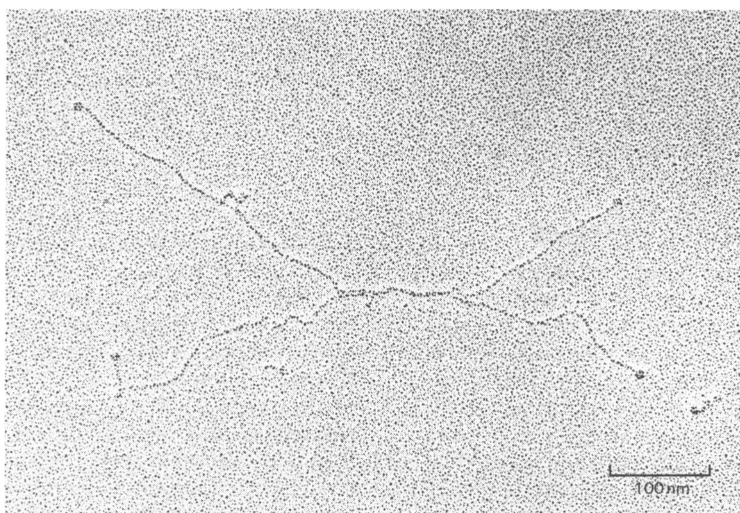

Fig. 2. *Electron micrograph of a type IV collagen 'spider' after rotary shadowing*
The type IV collagen used in this microscopy was prepared from an acetic acid extract of human placenta and shows no evidence of proteolytic attack.

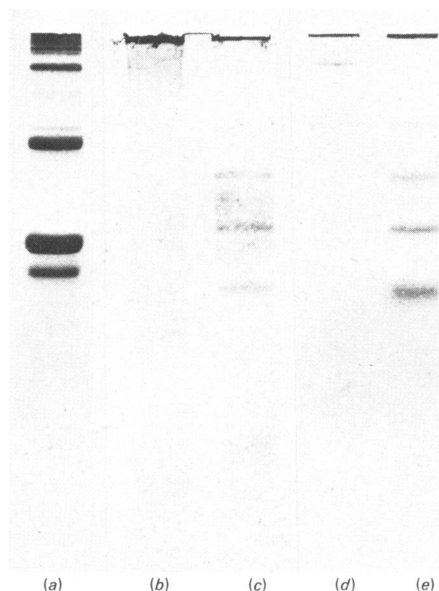

Fig. 3. *SDS/polyacrylamide-gel analysis of type IV collagen preparations*
Type IV collagen was prepared from NaB^3H_4-reduced and fresh human placenta by the procedures described in the Materials and methods section. The Figure shows samples of (*a*) human type I collagen, (*b*) and (*c*) type IV collagen isolated by pepsin treatment of NaB^3H_4-reduced human placenta (see the Materials and methods section), and (*d*) and (*e*) type IV collagen isolated by pepsin treatment of fresh human placenta (see the Materials and methods section). The samples were electrophoresed in 5.5% (w/v) acrylamide gels, both in the presence (tracks *c* and *e*) and absence (tracks *b* and *d*) of 2-mercaptoethanol.

mercaptoethanol; the resistance of these fragments from placenta clearly indicates the presence of covalent cross-links.

The salt-soluble fraction obtained from both NaB^3H_4-reduced and non-reduced placental type IV collagen after prolonged pepsin digestion contained the 'long-form' 7S domain of the molecules as revealed by electron microscopy (Fig. 5*b*). The 'long-form' 7S preparation from NaB^3H_4-reduced placenta was particularly pure, exhibiting a single high-M_r band (250000) that was unaffected by 2-mercaptoethanol reduction (Figs. 4*f* and 4*g*). The same 2-mercaptoethanol-resistant high-M_r component was observed in the preparation derived from untreated placenta corresponding to the 'long-form' 7S fragment. However, this sample was contaminated with some low-M_r material derived from the pepsin-cleaved helical domains and which showed up on gels both before and after reduction with 2-mercaptoethanol.

Cross-link analysis of type IV collagen reduced with NaB^3H_4 after extraction and purification

Type III and type IV collagen were prepared from human placenta to purity (as assessed by analysis on SDS/polyacrylamide gels) and a known weight of each was reduced with NaB^3H_4. Analysis of the acid hydrolysates of the two samples on ion-exchange columns developed with pyridine/formate buffers showed the presence of the reduced oxo-imine dihydroxylysinonorleucine in type III. When type IV collagen was purified from placenta and reduced, a recognizable and quantifiable dihydroxylysinonorleucine peak was obtained (Fig. 6*a*). No reduced aldimine, hydroxylysinonorleucine, was seen in these placenta preparations. It is worth recording that freeze-drying of the type IV preparation decreased the already low amount of ^3H incorporated into cross-links to a barely detectable level. The type III collagen sample showed only limited amounts of reduced hexosyl-lysine components, whereas in contrast the type IV collagen sample was rich in these residues.

Cross-link analysis of type IV collagen purified from NaB^3H_4-reduced placenta

When type IV collagen was prepared from NaB^3H_4-reduced placenta, acid hydrolysates of the protein were shown to contain the reduced oxo-imine dihydroxylysinonorleucine, as well as high proportions of reduced hexosyl-lysine components (Fig. 6*b*).

We also assayed the cross-link content of all other collagen types obtained as pure proteins from the reduced placenta for comparison with the type IV preparation. Table 1 shows a comparison of the ^3H incorporation into cross-links in collagen types I, III, IV (7S), IV (helices) and V. It should be noted that the different collagen types in human placenta were uniformly and reproducibly found to contain dihydroxylysinonorleucine as the predominant cross-link. It can be seen that the relative amount of this cross-link in the different collagen types is quite variable. The highest level of ^3H incorporation into dihydroxylysinonorleucine was seen in type III collagen. If this value is set at 100% for comparative purposes, the type I and type V cross-link ^3H incorporations are 62 and 89% respectively. These values may reflect decreased cross-linking or slower turnover and, thus, lower synthesis rates of these two collagens as compared with type III.

Type IV showed the lowest ^3H incorporation into hydroxylysino-5-oxonorleucine (Table 1), whether reduced before or after pepsin extraction. Despite the low level of incorporation, significant amounts of dihydroxylysinonorleucine could be identified in hydrolysates in both the 7S fragment and the helical (legs) region (Fig. 7). The cross-link

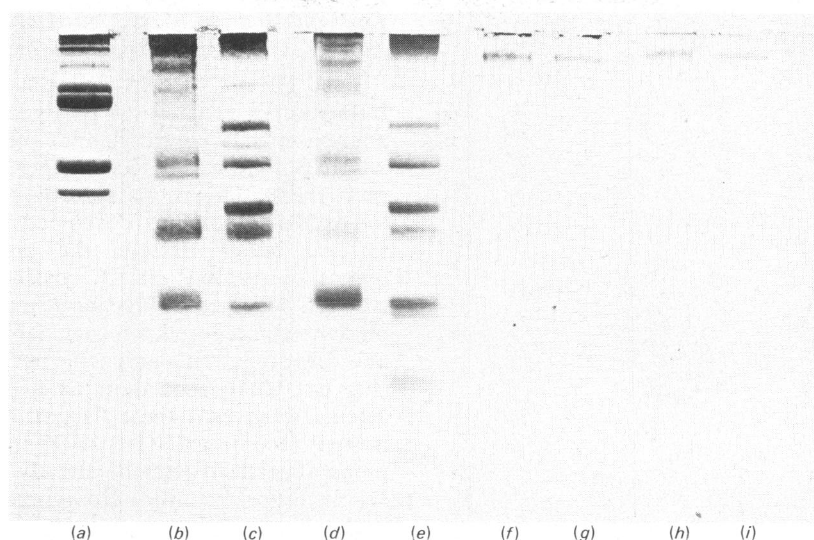

Fig. 4. *SDS/polyacrylamide-gel analysis of pepsin-derived type IV collagen fragments*
'Long-form-7S' and 'leg' fragments were prepared from the type IV collagen samples illustrated in Fig. 3 by an extended pepsin digestion described in the Materials and methods section. The Figure shows samples of (*a*) human type I collagen, (*b*) and (*c*) helical ('leg') region of type IV collagen from NaB^3H_4-reduced placenta, (*d*) and (*e*) helical ('leg') region of type IV collagen from fresh human placenta, (*f*) and (*g*) 7S region of type IV collagen from NaB^3H_4-reduced human placenta and (*h*) and (*i*) 7S region of type IV collagen from fresh human placenta. All of the type IV collagen fragments were produced by extended pepsin digestion (see the Materials and methods section). The samples were electrophoresed in 5.5% (w/v) acrylamide gels, both in the absence (tracks *a*, *b*, *d*, *f* and *h*) and presence (tracks *c*, *e*, *g* and *i*) of 2-mercaptoethanol.

peak in the legs region (Fig. 7*b*) may be partly due to contamination of 7S, but, on the basis of the level of 7S in the rotary-shadowing micrographs, its contribution is unlikely to be large.

Comparison of the analysis of NaB^3H_4-reduced glucosylated components in the 7S and helical type IV fractions shows that few hexosyl-lysine residues were present in the former sample (Fig. 7*a*). In the salt-insoluble 'leg' fraction, however, a high incorporation of ^3H into the hexosyl-lysine components was seen (Fig. 7*b*).

Finally, analyses of purified type IV collagen preparations for the putative stable cross-link pyridinoline were negative. As the sensitivity of the immunoassay used is in the 2pmol range, it seems certain that none of this component is present in type IV collagen.

Cross-link changes during aging of intact basement-membrane collagen

NaB^3H_4-reduced washed anterior lens capsules from 15-day and 21-day chick embryos were hydrolysed in acid and analysed for cross-link components by ion-exchange chromatography in pyridine/formate buffers. The ratio of the amounts of reduced hexosyl-lysines to dihydroxylysinonorleucine increased from 1.1 in the 15-day sample to 1.9 in the 21-day sample, indicating rapid maturation of the collagen (Heathcote *et al.*, 1980).

Discussion

We were able to duplicate previous work (Timpl *et al.*, 1981; Kuhn *et al.*, 1981) in extracting whole type IV collagen tetramers from washed placental cotyledons with 0.5M-acetic acid in the absence of proteinase inhibitors. These molecules appeared to be complete and, on electron-microscopic examination, showed all four major domains previously described, namely 7S, NC2, helical 'legs' and NC1.

We were not able to solubilize any type IV collagen from washed placenta with solutions containing 6M-urea and 10mM-dithiothreitol, demonstrating that both *N*- and *C*-termini of the molecules must be linked into the basement-membrane collagen matrix by intermolecular bonds other than disulphide bridges. This is supported by the increased solubility of lathyritic EHS-sarcoma basement membrane, from which type IV collagen may be extracted with 6M-guanidinium chloride and 10mM-dithiothreitol (Kleinman *et al.*, 1982).

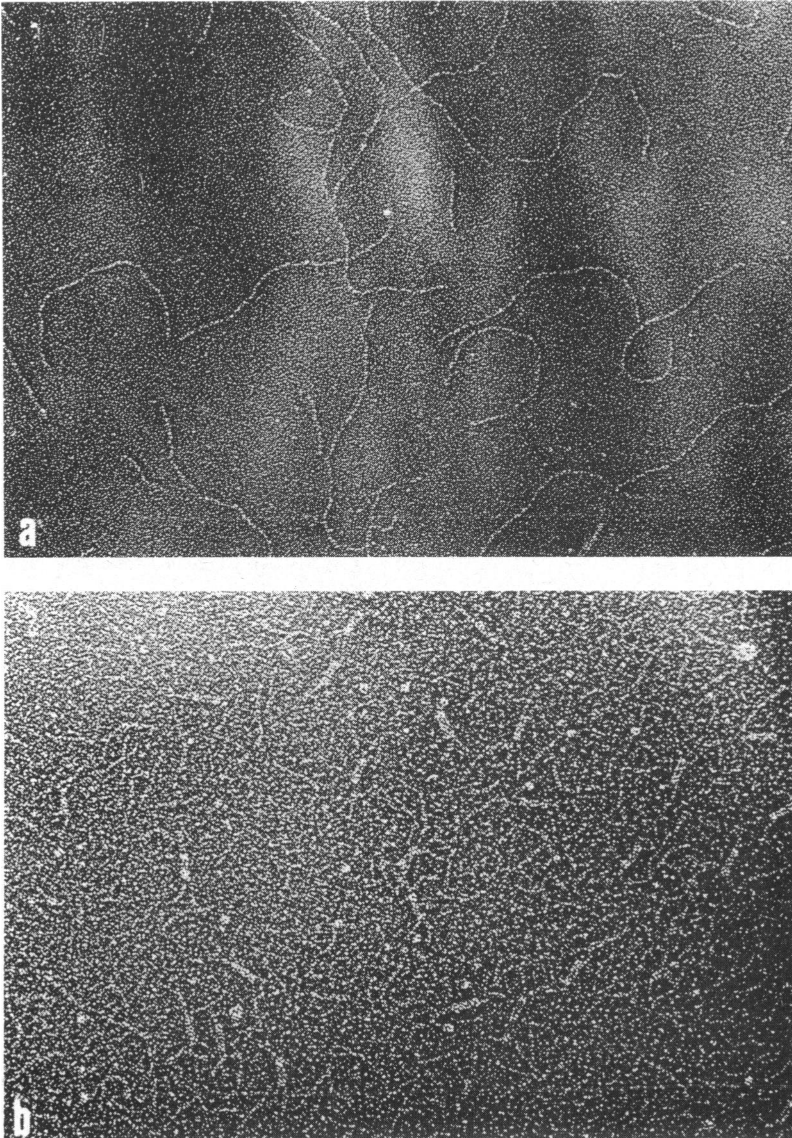

Fig. 5. *Electron micrographs of type IV collagen fragments*
Type IV collagen fragments were produced, prepared and rotary-shadowed before electron microscopy as described in the Materials and methods section. a, Helical ('leg') domains from type IV collagen prepared from unreduced human placenta; b, long-form 7S domains from type IV collagen prepared from unreduced human placenta.

By using extended pepsin digestion with fresh placenta and NaB^3H_4-reduced placenta we were able to locate sites of lysine-derived intermolecular cross-linking of type IV collagen in both the 7S domain and 'legs' region of the molecule. This procedure of reducing the whole placenta was necessary to ensure detection of any aldimine cross-link, which would be cleaved under the acid conditions of the pepsin digestion.

Both domains were found to contain the reduced oxo-imine cross-link dihydroxylysinonorleucine as well as reduced hexosyl-lysine components. The reduced aldimine cross-link, hydroxylysinonorleucine, could not be detected in these preparations. No other reducible compounds were present in significant proportions. ^3H incorporation into reduced oxo-imine in the 7S and helical 'leg' preparations, compared with other types of collagen (reduced under identical conditions in the same placenta sample at the same time), was low,

Table 1. *Comparison of ^{3}H incorporation into oxo-imine cross-links in different collagens prepared to purity from $NaB^{3}H_4$-reduced human placenta*

Collagen type	Radioactivity in oxo-imine (c.p.m./mg of collagen)	Percentage of type III collagen
I	524	62
III	851	100
V	756	89
IV (7S)	148	17
IV ('legs')	233	27

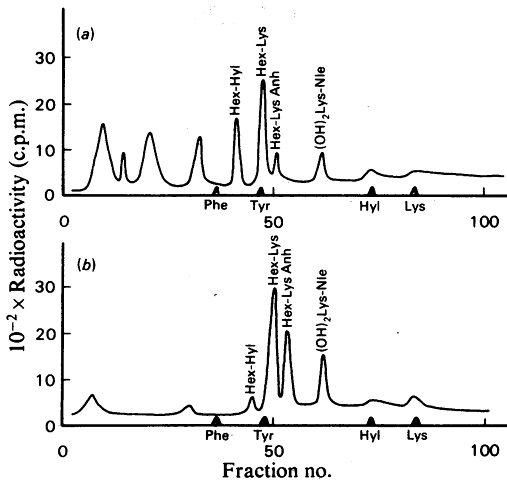

Fig. 6. *Ion-exchange chromatography of ^{3}H-labelled reduced amino acids and cross-links from type IV collagen*
Profiles of radioactivity were produced after ion-exchange chromatography in volatile buffers (see the Materials and methods section) of acid hydrolysates of (*a*) type IV collagen $NaB^{3}H_4$-reduced after purification and (*b*) type IV collagen prepared from $NaB^{3}H_4$-reduced human placenta. Abbreviations used: Hex, hexosyl-; Anh, anhydride; $(OH)_2$Lys-Nle, dihydroxylysinonorleucine.

about 17 and 27% of the incorporation seen in type III collagen respectively.

A possible explanation for the relatively low number of cross-links observed in these experiments is that the type IV collagen molecule contains less cross-links per thousand residues than any other type of collagen. However, this explanation does not seem likely if the 7S domain is considered. In contrast with the 7S fragment from lathyritic tissue, cross-linked 7S does not break down to low-M_r components, indicating that all four 7S chains must be linked by lysine-derived cross-links. Therefore a minimum of three lysine-

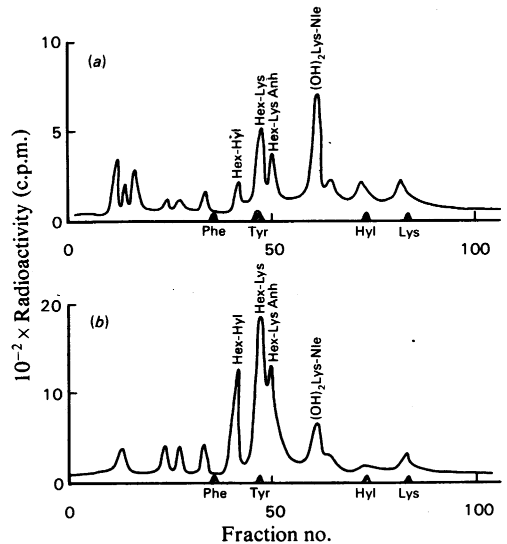

Fig. 7. *Ion-exchange chromatography of ^{3}H-labelled reduced amino acids and cross-links from pepsin-derived fragments of type IV collagen derived from $NaB^{3}H_4$-reduced human placenta*
Profiles of radioactivity were produced after ion-exchange chromatography in volatile buffers of acid hydrolysates of (*a*) long-form 7 s and (*b*) helical 'leg' fragments. See the Materials and methods section for details. For abbreviations, see Fig. 6.

derived cross-links per 'long-form 7S' unit ($M_r \sim 250000$) would be necessary to stabilize the complex against the effects of reducing agents such as 2-mercaptoethanol. One to two cross-links per molecule of fibrous collagen (M_r 285000) has been established biochemically, i.e. approx. 0.3–0.7 cross-links per 1000 residues. In comparison, the density of cross-linking in the 7S unit would have to be at least 1.1 cross-links per 1000 residues. This conclusion rules out the likelihood of the 7S unit requiring less cross-links than the fibrous collagens.

A more reasonable hypothesis is that type IV collagen contains non-reducible covalent cross-links as well as oxo-imines and disulphide bonds. Our data from chick lens capsule demonstrate that newly-laid-down type IV collagen contains much higher quantities of oxo-imine and that these cross-links 'disappear' rapidly during maturation. We found that the ratio of reduced oxo-imine to hexosyl-lysine residues fell from 0.9 in the 15-day lens-capsule samples to 0.5 in those from 21-day chick embryos. In terms of the total ^{3}H incorporated, the number of oxo-imine cross-links detectable by this method fell by approx. 32% over this short period. This evidence strongly suggests that the oxo-imines are converted into a non-reducible,

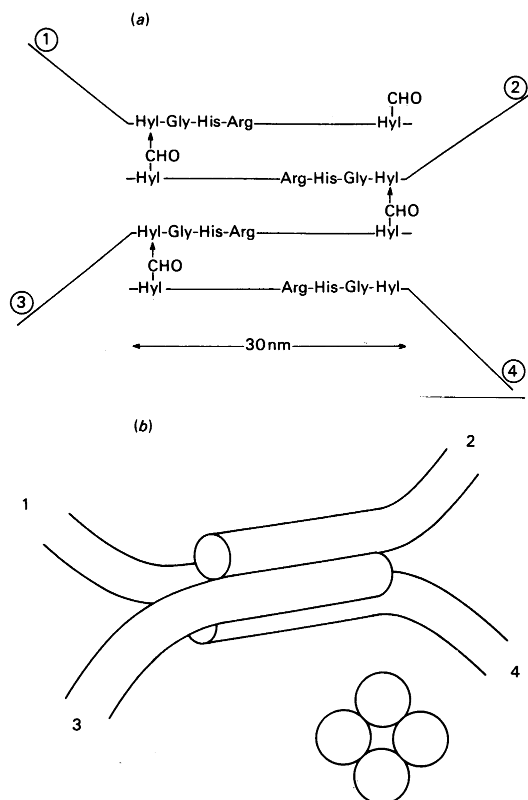

Fig. 8. *Diagram of a cross-linked 7S domain*
(*a*) The Figure shows how 'hydroxylysine aldehydes' formed at the *N*-termini may interact with adjacent hydroxylysine residues, which themselves form part of the conserved Hyl-Gly-His-Arg sequence, to form reducible oxo-imine cross-links. Each of the four molecules (1–4) are triple helices containing a maximum of three aldehyde groups for reaction. (*b*) Illustration of a possible packing scheme for the 7S domain in which all four chains are in close juxtaposition, allowing maximum cross-linking.

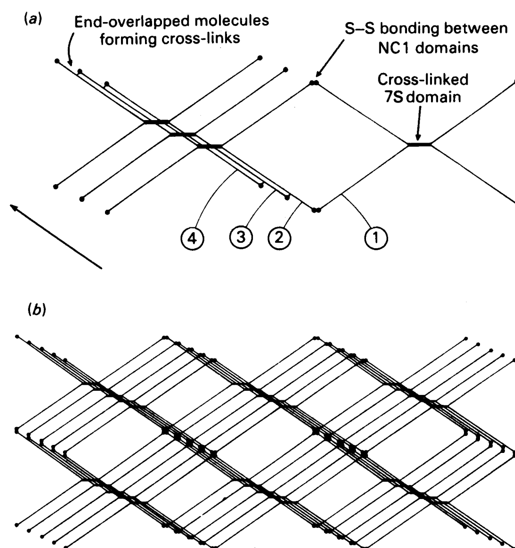

Fig. 9. *Schematic representation of a possible end-overlapped type IV collagen matrix*
(*a*) Tetramers ('spiders') 1 and 2 are linked by S–S bonds through juxtaposed NC1 domains and form part of a 'chicken-wire' lattice (see Fig. 1) in the plane of the basement membrane. Tetramer 3 is superimposed upon tetramer 2 in the same plane and forms part of a second 'chicken-wire' lattice. It, and all other tetramers in this second lattice, is staggered with respect to tetramer 2 (and all other treatments in the first lattice) by the distance required to give an end-overlap in the direction indicated by the arrow. Tetramer 4 is similarly staggered with respect to tetramer 3. In this way, layers of 'chicken-wire' lattices may be built up in the plane of the basement membrane in a staggered fashion to allow cross-linking. (*b*) A section of five 'chicken-wire' lattices overlaid one upon the other as described in (*a*). Note that both 7S and NC1 domains are placed at regions of high α-chain density to facilitate the formation of complex non-reducible cross-links (see the text).

possibly polyvalent form, as has been previously suggested in the maturation of type I collagen (Robins *et al.*, 1973; Light & Bailey, 1979, 1980*a*, *b*). However, unlike type I collagen, the rate of formation of the non-reducible cross-links from the oxo-imines in type IV appears to be extremely fast, as evidenced by our findings in the chick lens-capsule preparations and those of Heathcote *et al.* (1980) with bovine lens capsule. Rapid maturation of the cross-links is only made possible because the basement-membrane collagen appears to turn over very slowly (Rafferty & Goossens, 1978; Heathcote *et al.*, 1980).

The question of what form the 'mature' non-reducible 7S and 'legs' cross-links take remains unresolved. The tervalent intermolecular collagen cross-link pyridinoline has been proposed as the 'mature' cross-link (Fujimoto *et al.*, 1977, 1978; Eyre & Oguchi, 1980). It has been shown to be associated only with highly hydroxylated collagen, e.g. cartilage and bone, and to form from one or two oxo-imine cross-links (Eyre & Oguchi, 1980; Robins, 1983*b*,*c*). It might have been expected, therefore, that type IV collagen would contain pyridinoline, but our concerted attempts to demonstrate the presence of this compound, either by classical amino acid analysis or using a specific immunoassay, proved negative. We may conclude, therefore, that the oxo-imine cross-links of type IV collagen mature to a different unknown non-

reducible form. The most likely proposal is that it is similar to the unknown 'mature' cross-link of the fibrous collagens (Light & Bailey, 1980*b*).

A recent report (Dieringer & Glanville, 1983) described the presence of *intra*molecular aldols in the helical 'leg' of the type IV molecule located 45 nm from the 7S domain. Such cross-links would not be expected to contribute to the stabilization of the 7S domain. However, it does indicate the presence of 'lysine aldehyde' (2,6-diaminohexanaldehyde) molecules which might even be capable of alternative interaction.

Although the precise location of the cross-link must await amino acid sequence data, we can interpret our finding in the light of the current model of type IV collagen. The collagenous matrix of basement membrane is stabilized by the same cross-linking mechanism previously described for the fibrous collagens. The type IV molecule contains 'lysine aldehyde' molecules at the *N*- and *C*-termini and, after aggregation of four of these into the 'spider' (Dureau *et al.*, 1983), the *N*-terminal aldehydes stabilize the 7S domain through the oxo-imine cross-link.

By analogy with the cross-linking of fibrous collagens, this conclusion leads to an important prediction. Types I, II and III collagen possess conserved sequences of the tetrapeptide Hyl-Gly-His-Arg approx. 30 nm from each end of the molecule. It is this hydroxylysine which reacts with end-overlapped 'lysine aldehyde' or 'hydroxylysine aldehyde' molecules at the *N*- and *C*-termini of adjacent molecules to form aldimine or oxo-imine cross-links. Fig. 8(*a*) shows a schematic illustration of the 'long-form' 7S domain. As can be seen, 7S itself is 30 nm long, so that *N*-terminal 'hydroxylysine aldehyde' molecules must react with adjacent hydroxylysine molecules which may be expected to form part of the conserved Hyl-Gly-His-Arg sequence. We may predict, therefore, that, provided the primary structure is reasonably well conserved in type IV collagen, the *C*- to *N*-terminal sequence (as illustrated in Fig. 8) will contain the Hyl-Gly-His-Arg tetrapeptide approx. 30 nm from the *N*-terminal end of the molecule and the cross-links will be located as indicated. Furthermore, by 'wrapping-up' the 7S domain by interlocating the four adjacent helices (as shown in Fig. 8*b*) the final free aldehyde and hydroxylysine may be brought into juxtaposition so that all active groups will react to form cross-links.

This argument can also be applied to the NCl domains in that aldehydes formed by lysyl oxidase in the non-helical-region *C*-terminus should interact with hydroxylysine molecules in a helical segment. By analogy with fibrous collagen we may expect, as explained above for the 7S domain, that the NCl aldehydes will overlap another Hyl-Gly-His-Arg sequence in an adjacent molecule at a point some 30 nm from the beginning of the triple helix at the *C*-terminal end. This would therefore facilitate the *C*-terminal-to-*C*-terminal cross-linking which we have observed.

Consideration of *C*-terminal-to-*C*-terminal cross-linking indicated by our results raises certain difficulties for the 'chicken-wire' structure (Fig. 1). To accommodate this type of cross-linking the adjacent 'spiders' must be end-overlapped, hence, a linear array of side-to-side 'spiders' can be linked covalently. However, linking these ribbons of stabilized 'spiders' laterally will now prove impossible if the same end-overlapped mechanism is invoked. It would seem that the 'chicken-wire' structure alone may be too simple to fulfil these cross-linking requirements.

Similarly (as we have shown that an unknown non-reducible cross-link forms from the oxo-imines initially laid down in the type IV collagen network), it is necessary that the chains cross-linked by the oxo-imine in one plane must overlay another chain in a second plane such that a polyvalent cross-link may then form. This is analogous to the acknowledged situation in type I collagen where molecules are staggered with respect to one another in one dimension but in register in the second to facilitate formation of complex, non-reducible cross-links from bivalent reducible bonds (Light & Bailey, 1980*a*,*b*). These two requirements, for an overlap system for the formation of oxo-imines and for close associations between these oxo-imines and residues of collagen chains in the next plane, are fundamental and must be fulfilled if cross-linking is to be controlled and complete. We propose therefore that a superimposed but end-overlap staggered mesh-work is more probable for the matrix structure of type IV collagen. Fig. 9 illustrates one variation of this type of model; however, the elucidation of the exact three-dimensional structure of the type IV collagen matrix must await further research.

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