Self-Assembly of Basement Membrane Collagen†

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ABSTRACT: The in vitro self-assembly of murine type IV collagen was examined by using biochemical and morphological techniques. Dimeric collagen undergoes a rapid and reversible thermal gelation at neutral pH without an appreciable lag period. The process is seen to be concentration dependent and inhibited by 2 M urea. The formed complex can be visualized by electron microscopy rotary shadowing as an irregular polygonal lattice network with extensive side by side associations within the collagenous triple-helical part of the molecules, two and three strands thick. Measurements on the matrix suggest a median stagger dimension of 170 nm, one-fifth the length of a dimer. The conversion of pepsin-generated monomers into N-terminally bound tetramers can also be demonstrated in vitro. This process is also concentration dependent and inhibited and reversed by 2 M urea but is thermally irreversible and occurs at a slow rate relative to the lateral associations. These tetramers can be seen by rotary shadowing as four-armed "spider" structures. It is proposed that lateral associations, by virtue of their faster rate of formation, precede 7S bond formation, and several models for the assembly of basement membrane collagen are discussed.

Type IV collagen, a major component of basement membranes (Kefalides, 1973), is a structurally unique collagen (Bornstein & Sage 1980; Crouch et al., 1980; Heathcote et al., 1978; Minor et al., 1976; Trygvason et al., 1980) composed of two different types of constitutive chains. Basement membranes do not contain cross-striated fibers and, unlike the interstitial collagens, type IV collagen does not appear to form such fibers in vitro under physiologic salt conditions (Schwartz et al., 1980; Veis & Schwartz, 1981). This collagen possesses a number of distinct chemical features. The major triple-helical domain contains frequent interruptions of the regular collagenous Gly-X-Y triplet by sequences without this feature which are seen as flexible and protease-sensitive sites (Bornstein & Sage, 1980; Schuppan et al., 1980; Timpl et al., 1981; Hofmann et al., 1984). At the amino terminus of the triple-helical monomer, there is a disulfide-rich domain of about 300 Å which participates in binding to three other amino-terminal (N-terminal)[1] ends to produce a tetramer (Timpl et al., 1981; Kühn et al., 1983; Duncan et al., 1983). The tetrameric domain has been isolated by proteolysis to yield a temperature-stable covalently cross-linked fragment with an apparent molecular weight of 200 000–360 000 and a Svedberg value of about 7 S (Risteli et al., 1980). At the C-terminal end of the molecule (Fessler & Fessler, 1982), there is a large globular domain with a molecular weight of about 75 000 (Timpl et al., 1981) which participates in bonding to the globular domain of another molecule. Using these two end-region bonds, it is possible to hypothesize an arrangement of type IV collagen in a two- and even three-dimensional matrix (Timpl et al., 1981).

However, there are a number of reasons to suspect that such a model represents an oversimplification. First, it denies the existence of side by side collagenous associations even though some 75–80% of the amino acid sequence is collagenous in nature. Second, it produces a structurally weak matrix with flexible single strands which would be, on its own, a poor candidate as a skeletal framework for the other basement membrane proteins. Third, it results in an enormous potential pore size with a side dimension of over 800 nm.

Type IV collagen, purified from lathyritic murine tumor sources, was examined with respect to self-assembly. The goals were to see if noncovalent associations precede covalent linkage and therefore determine specificity of binding, to look for a third type of association (in particular, lateral or collagenous binding), and finally to see if a model for the assembly of the matrix could be formulated.

Experimental Procedures

Preparation of Type IV Collagen. (A) EHS. The collagen was prepared from 200–400 g of lathyritic Engelbreth-Holm-Swarm (EHS) mouse tumor by the method of 2 M guanidine–2 mM dithiothreitol (DTT) extraction as described (Kleinman et al., 1982) followed by further purification at 4 °C using differential salt precipitation, DEAE-cellulose chromatography, and, where indicated, gel filtration as follows: The guanidine extract, in a total volume of about 1 L, was dialyzed into 10 L of 1.7 M NaCl–50 mM Tris-HCl, pH 7.4, with 2 mM DTT, 1 mM EDTA, and 0.1 mM PMSF and then centrifuged at 12 000 rpm for 1 h. The pellet was redissolved in 2 M guanidine, 50 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM EDTA, and 0.1 mM PMSF (buffer A), dialyzed into 4 M urea, 0.25 M NaCl, and 50 mM Tris-HCl, pH 8.6, with 1 mM DTT, 1 mM EDTA, and 0.1 mM PMSF with several buffer changes, and then passed down a 2.5 × 10 cm DEAE-cellulose (Whatman) column equilibrated in the same buffer. The unbound fraction was dialyzed into buffer A (used for storage on ice) and then centrifuged in a Beckman Ti60 ultracentrifuge rotor at 60 000 rpm for 90 min to remove aggregated material. The material as prepared was found by rotary shadowing to be about 80% dimeric on a mass basis. The dimeric fraction could be isolated by dialyzing into 2 M urea, 50 mM Tris-HCl, pH 7.4, 2 mM DTT, 5 mM glycine, 1 mM EDTA, and 0.1 mM PMSF (buffer B) and, following centrifugation to remove any new formed precipitate, chro-

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[1] Abbreviations: N terminal, amino terminal; C terminal, carboxy terminal; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride.

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matographed in 20–25-mL aliquots on a 5 × 95 cm Sephacryl S1000 (Pharmacia) column equilibrated in buffer B. The pooled fractions were concentrated in dialysis bags covered with Aquacide IIA (Calbiochem) and dialyzed back into buffer A for storage. The estimated overall recovery from 400 g of tumor was 1 g. The collagen, following purification through DEAE-cellulose and ultracentrifugation, was found to contain less than 0.02% laminin and less than 0.1% type I collagen contamination by ELISA inhibition assay.

(B) PF-HR9 Medium Collagen. PF-HR9 cells, maintained in culture flasks as described (Bächinger et al., 1982), were biosynthetically labeled with [^{35}S]methionine (New England Nuclear, 1110 Ci/mmol) for 24 h in Dulbecco's modified essential medium without methionine but with 50 μg/mL ascorbate and 2% fetal calf serum. The medium was precipitated with ammonium sulfate to 45% saturation followed by purification on DEAE-cellulose and then by gel filtration as described (Bächinger et al., 1982) except that Sephacryl S1000 was used instead of A50m agarose and in the absence of sucrose. This collagen, after ion-exchange chromatography, was found to be almost entirely monomeric by rotary shadowing.

^{125}I Iodination of EHS Type IV Collagen. Two milliliters of EHS type IV collagen was dialyzed against 4 L of 2 M urea–50 mM Tris-HCl, pH 7.35 at 4 °C, and then centrifuged at 40000 rpm for 20 min in a Ti65 rotor. Two milliliters of supernatant was mixed with 5 mCi of carrier-free Na^{125}I (Amersham Searle), 50 μL of a 1 mg/mL solution of lactoperoxidase (Sigma) in 10 mM sodium phosphate, pH 7.4, containing 120 mM sodium chloride (PBS), and 100 μL of 0.003% H_2O_2, and the solution was incubated at 24 °C for 20 min. Twenty microliters of 0.2 M DTT was added, and the sample was chromatographed on a 20-mL Sephadex G25 (medium) column (Pharmacia) equilibrated in buffer B to remove the bulk of free iodine. The peak void fractions were pooled and rechromatographed at 4 °C in a 1.5 × 95 cm Sephacryl S1000 column in buffer B. The pooled dimeric peak (running at 0.55 of the column volume) was then concentrated with Aquacide, dialyzed against buffer A, and stored on ice.

Limited Pepsin Digestion of Type IV Collagen. EHS type IV collagen at 0.6–1 mg/mL, mixed with trace amounts of ^{125}I-labeled type IV collagen dimer, was dialyzed against 0.1 M acetic acid (pH 3) at 4 °C and then mixed with pepsin (Worthington) at an enzyme:substrate ratio of 1:50. After incubation at 28 °C for 70 min, this material was placed on ice, mixed with pepstatin A (Sigma), dialyzed into buffer B, and chromatographed on a 2.5 × 95 cm or 5 × 95 cm Sephacryl S1000 column in buffer B. The monomeric peak was pooled, concentrated with Aquacide as previously described, dialyzed into buffer A, and stored on ice.

Falling Ball Relative Viscosity Measurements. One hundred microliter glass micropipets (Boralex) were filled with protein samples, capped at one end, and incubated for various times at 28 °C. The tubes were then fixed at a 45° angle, and the rate at which a 0.64-mm 440-C steel ball (New England Miniature Ball Co.) passed through three tubes was measured for each time point (Griffith & Pollard, 1978; Fowler & Taylor, 1980).

Turbidity Measurements. Collagen solutions were dialyzed at 4 °C into PBS with 0.2 mM DTT and 0.1 mM EDTA (neutral phosphate buffer) or other buffer, when indicated, and centrifuged at 40000 rpm at 3 °C in a Ti65 rotor for 15–20 min or at 14000 rpm for 30 min. The supernatants were placed in prewarmed quartz cuvettes at 28 °C (maintained with a water jacket), and the absorbance at 360 nm was followed over time. For thermal reversibility studies, the cuvette chamber was cooled with circulating ice-cooled water to a cuvette measured temperature of 10–11 °C.

Rate Zonal Velocity Sedimentation. Five to twenty percent (w/v) linear sucrose gradients (12 mL) in various buffers were prepared on 50% sucrose cushions (0.35 mL). Sample volumes were generally 0.2 mL. Centrifugation was performed by using an SW 40Ti rotor in a Beckman L5-65 ultracentrifuge either at 3 °C for 22 h at 40000 rpm or at 28 °C for 13.5 h at 38000 rpm. Cytochrome *c* (1.8 S), bovine serum albumin (4.3 S), aldolase (7.35 S), and catalase (11.3 S) (Sigma) were used as standards to determine apparent *s* values and run in parallel gradient tubes at the two temperatures and different buffers. Fractions (0.2 mL) were collected by drop counting and measured with respect to absorbance or radioactivity.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Samples of collagen were electrophoresed on 5% acrylamide slab gels 1.5 mm thick in the presence of NaDodSO$_4$ with or without 1.5% 2-mercaptoethanol as described by Laemmli (1970).

Rotary Shadowing. The rotary shadowing method was adapted from Shotten et al. (1979). Briefly, collagen samples were incubated in either 0.1 M acetic acid (pH 3), PBS (pH 7.35), 0.2 M ammonium acetate (pH 7.5) or 50 mM Tris-HCl, pH 7.9, with 1.2 M NaCl (TBS) and then diluted into 0.2 M acetic acid or 0.15–0.2 M ammonium acetate. In some cases, the samples were then mixed with an equal volume of glycerol. Samples, at a final concentration of 10–15 μg/mL, were then sprayed onto freshly cleaved mica sheets at a distance of about 40 cm and shadowed at a 6° angle in a vacuum chamber at 2 × 10^{-5} torr with carbon/platinum. The replicas were transferred onto uncoated copper grids and viewed with a Philips 300 electron microscope at 60 kV using a 30-μm objective operative. Pictures were taken on Kodak 4489 EM film.

Measurement of Collagen Lengths. Rotary shadow replicas were routinely photographed in the electron microscope at 24000× magnification. Prints of electron microscope (EM) photographic negatives were enlarged 15-fold (363000× final magnification). Multiple length measurements were made by using a Zeiss Videoplan computer with a digitizer measuring tablet attachement and were plotted in histogram form by using a (Y)1 VIDEOPLAN program.

Determination of Protein Concentration. Protein concentrations of standard EHS collagen were determined on a dry weight basis after dialysis into 0.1 M acetic acid. The protein concentration of other samples was determined by a competition enzyme linked immunosorbant assay (ELISA) using affinity-purified rabbit anti-mouse type IV collagen antibody kindly provided by Dr. Joseph Madri (Yale University School of Medicine). The logarithmic portion of the dilution curve was analyzed by linear regression for both standard and sample solutions.

Results

Characterization of Type IV Collagen by Gel Electrophoresis and EM Rotary Shadowing. EHS type IV collagen, when subjected to electrophoresis on NaDodSO$_4$–polyacrylamide gels (Figure 1A), was seen to migrate predominantly (about 60%) as a doublet (α1 and α2 chains) at 185000 and 170000 daltons, respectively (determined from a type I collagen standard). The two bands are present in a 2:1 ratio. The remainder of the material (about 40%) is found in bands of higher molecular weight which appear to represent the β and γ components of type IV collagen. In contrast, the majority of the type IV collagen derived from the medium of the HR9 cell line migrates in the α1 and α2 positions, presumably due

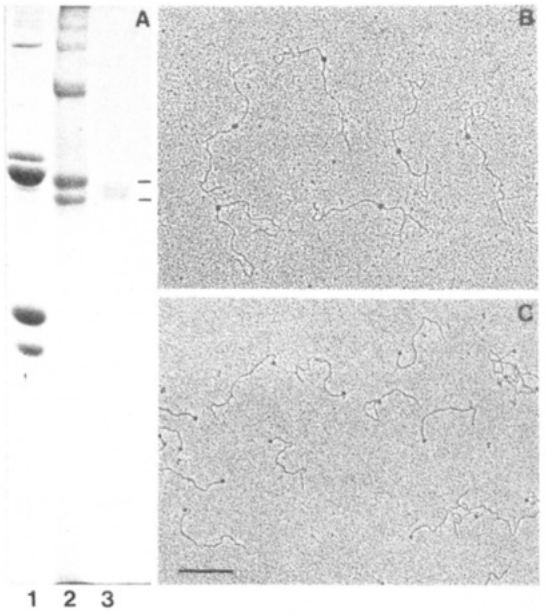


FIGURE 1: Characterization of type IV collagen. (A) Characterization by $NaDodSO_4$–polyacrylamide gel electrophoresis: Coomassie blue stained slab gels of acid-soluble mouse type I collagen (kindly provided by Joseph Madri) (lane 1), guanidine–DTT-extracted EHS type IV collagen purified through DEAE-cellulose (lane 2), and PF-HR9 type IV collagen isolated from cell culture medium and purified through DEAE-cellulose (lane 3). Parallel lines to the right of the gel indicate the α1 and α2 positions for type IV collagen. (B and C) Characterization of type IV collagen by rotary shadowing. EHS type IV collagen dimer preparation prepared by purification on Sephacryl S1000 was shadowed following spraying onto mica sheets in 0.1 M ammonium acetate in 50% glycerol at about 10 μg/mL (B). The majority of fields were as shown. PF-HR9 type IV collagen, derived from cell culture medium and purified by DEAE-cellulose chromatography, was shadowed by using the same buffer conditions (C). Bar equals 200 nm.

to less cross-linking. The small differences in migration positions between the two collagens may be due to differences in posttranslational modification, or they simply represent variation from one sample to the other, since it has been observed occasionally also for EHS-derived type IV collagen. These differences in electrophoretic mobility are not reflected in measurable differences in the lengths of monomeric molecules (see below).

The EHS IV collagen of a typical preparation is about 80% dimeric on a mass basis when analyzed by the rotary shadowing method and is nearly 100% dimeric following gel filtration on Sephacryl S1000 (Figure 1B). The HR9 collagen, when derived from the medium, is almost entirely monomeric (Figure 1C). The monomeric (i.e., one triple-helical molecule on the average composed of two α1 and one α2 polypeptide chains) EHS collagen length is 424 ± 24 nm (n = 84) and is statistically of the same length as the HR9 collagen at 412 ± 69 nm (n = 66). As will be discussed later, the dimeric and monomeric forms are not interconvertible in vitro in our hands.

In Vitro Assembly of EHS Collagen into Large Complexes. When type IV collagen solutions at neutral pH are incubated at elevated temperature, the solutions apparently gel. The method of falling ball viscometry was used to measure increases in relative viscosity over time for type IV and type I collagens under physiologic salt conditions. EHS type IV collagen was dialyzed in the cold into neutral phosphate buffer at a concentration of about 0.8 mg/mL. When the temperature was raised to 28 °C, it was possible to demonstrate a gradual increase in viscosity (Figure 2A) without an obvious initial lag period. In contrast, acid-soluble rat type I collagen, analyzed at 0.1 mg/mL, developed a rapid increase in viscosity following a lag period of about 2 h. Both viscous solutions, when subjected to shear force (extrusion from the measuring pipet followed by refilling), lost their attained viscosity to the zero-time value, indicating a thixotropic property. Type I collagen reached a much higher maximum viscosity (13 s/cm) compared to type IV collagen (1 s/cm) even though it was at nearly one-tenth the concentration. Hence, both collagens were capable of increasing in viscosity but at different rates and to different extents.

The apparent formation of large molecular complexes could also be followed by turbidimetric analysis (Figure 2B,C), a method used for many years to study the fibrillogenesis of the interstitial collagens. EHS type IV collagen, at 1.2 mg/mL, was dialyzed into neutral phosphate buffer, centrifuged to reduce preformed aggregates, diluted to 0.1–0.6 mg/mL, and then placed in a quartz cuvette maintained at 28 °C. Turbidity increased over a several hour period and approached a plateau in 1–3 h. The maximal turbidity achieved was seen to be concentration dependent, and half-maximal turbidity was achieved at 30–40 min in the concentration range of 0.1–0.5 mg/mL. No lag period was appreciated unlike the reported observations of short lag periods for pepsin and acid-extracted lens capsule collagen (Schwartz et al., 1980; Veis & Schwartz, 1981). Cooling the cuvette to about 10 °C led to a gradual decrease in turbidity, indicating thermal reversibility of complex formation. At low concentrations, thermal reversibility was found to be complete. At high concentrations, a change of 15% was observed within the 24 h of the experiment. A comparison of the profile for turbidity development between acid-soluble type I collagen and EHS type IV collagen (0.2 mg/mL) is shown in Figure 2C. Mouse type I collagen exhibited the characteristic lag period followed by a rapid increase in turbidity (growth phase) and then a stabilization of turbidity (Plateau). The lag seen for mouse type I collagen by turbidity analysis was shorter than that observed by viscosity analysis for rat type I collagen. The difference may reflect a difference in collagen source since mouse collagen (at 0.1 mg/mL) exhibits a lag period of 1 h (at 28 °C) by falling ball viscometry. Type IV collagen, after ultracentrifugation sufficient to clear 50 S (reducing the initial absorbance almost to zero with essentially no aggregates greater than 5.4 S as determined by velocity sedimentation), rapidly increased in turbidity without apparent lag. Compared to type I collagen at 0.1 mg/mL, however, it attained a substantially lower (10-fold) maximum turbidity.

The thermally generated turbid material could be sedimented by centrifugation at 28 °C. Cold (0–3 °C) or acid (0.1 M acetic acid, pH 3) inhibited the conversion of soluble type IV collagen to precipitable material. EHS type IV collagen, containing trace amounts of ^{125}I label, was dialyzed into neutral phosphate buffer or 0.1 M acetic acid and then centrifuged at 3 or 28 °C (40 000 rpm for 20 min). Measurement of radioactivity in the supernatant and pellet revealed that a substantial portion of the collagen was pelleted if first warmed to 28 °C (41% at 0.38 mg/mL); however, in acetic acid, essentially no collagen was precipitated (1% at 0.38 mg/mL). If maintained for several hours at 3 °C, only small amounts of collagen could be sedimented (8%). Sephacryl S1000 purified dimeric collagen exhibited the same type of behavior at 0.3 mg/mL with 37% precipitated at 28 °C and 4% precipitated at 3 °C. The precipitated collagen had the same appearance as the collagen remaining in the supernatant when examined by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). The extent of conversion, as measured in direct sedimentation runs, was found to be con-

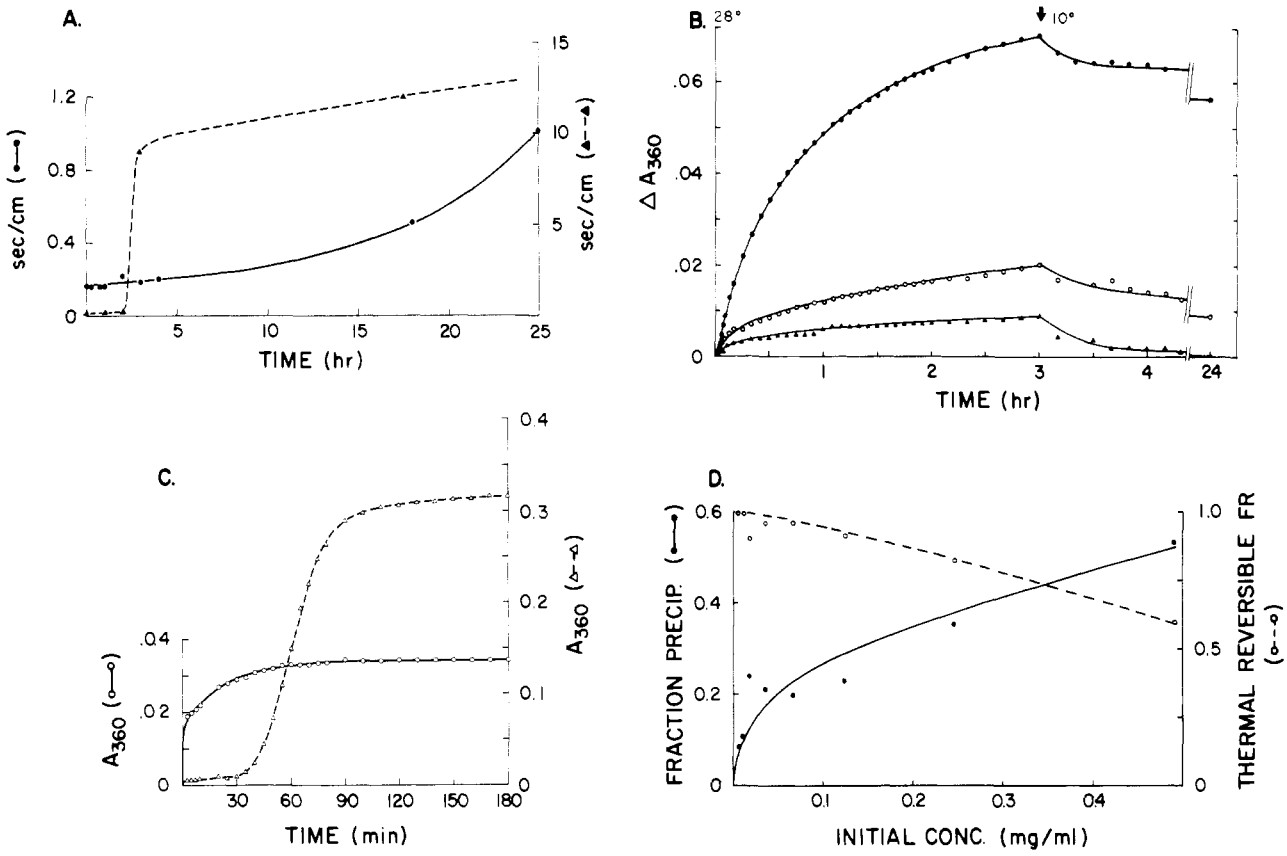


FIGURE 2: Changes in the physical characteristics of type IV collagen at 28 °C. (A) Falling ball viscometry. EHS type IV collagen was dialyzed into neutral phosphate buffer in the cold at about 0.8 mg/mL, drawn up into 100-μL pipets, and incubated at 28 °C for various periods of time. The rate of fall for a steel ball was measured as described in the text (solid line). As a control, the same procedure was carried out with rat acid-soluble type I collagen (kindly provided by Joseph Madri, Yale University School of Medicine) at 0.1 mg/mL (dashed line). When either solution was allowed to run out of the tube and then refilled into the same tube, the viscosity was found to decrease to the initial time value. (B) Turbidity, type IV collagen. The absorbance at 360 nm was measured as a function of time at 28 °C following dialysis of type IV collagen samples into neutral phosphate buffer in the cold at 0.55 (closed circles), 0.24 (open circles), and 0.12 mg/mL (closed triangles) following centrifugation at 14000 rpm for 30 min (B). Initial (zero time) turbidity values were subtracted and were 0.036 for 0.55 mg/mL, 0.021 for 0.24 mg/mL, and 0.011 for 0.12 mg/mL. After maintenance in cuvettes for 3 h, the cuvette holders were cooled down to about 10 °C, and the absorbance was followed over time. (C) Turbidity, type IV vs. type I collagen following ultracentrifugation. Type IV collagen (open circles) after dialysis into neutral phosphate buffer was centrifuged at 40000 rpm for 15 min at 3 °C (initial absorbance at 360 nm was 0.003). The turbidity profile for acid-soluble mouse type I collagen (open triangles) at 0.1 mg/mL is shown for comparison. (D) Concentration dependence of type IV collagen multimer formation. EHS collagen containing trace ^{125}I label was dialyzed into neutral salt buffer and then sedimented at 40000 rpm for 45 min at 3 °C. The supernatants were diluted by 2-fold serially, divided into 0.5-mL aliquots, and incubated at either 0 or 28 °C for 4 h. An additional series of aliquots was incubated at 28 °C for 4 h followed by 0 °C for 4 h (for determination of thermal reversibility). The samples were then sedimented at 28 °C (for 28 °C incubated samples) or 3 °C (for those samples whose final incubation was on ice) at 40000 rpm for 15 min. Three to six 10-μL aliquots were removed for determination of radioactivity before and after ultracentrifugation to determine the fraction precipitated. The fractions which sedimented in the cold only (average 0.08) were subtracted from the warm precipitate fraction to determine the net fraction precipitated at 28 °C (closed circles, solid line), and the fraction capable of thermal reversal (resolubilizing at 0 °C) was determined (open circles, dashed line) by subtracting from unity the net fraction which precipitated following cooling for 4 h.

centration dependent (Figure 2D) with up to 55% precipitable at 0.5 mg/mL. Since saturation had not been achieved at that concentration, one would predict even higher amounts to be precipitable at higher concentrations. However, concentrations above about 0.6 mg/mL in neutral phosphate buffer could not be readily achieved under the conditions used in this experiment in which the collagen was subjected to ultracentrifugation prior to thermal gelation. The extent of thermal reversibility, as measured by direct precipitation, was also shown to be a concentration-dependent process. Essentially complete reversibility of complex formation could be achieved at less than 0.2 mg/mL. At 0.5 mg/mL at 0–3 °C, some 65% of the aggregate could be reversed into a nonsedimentable form. Under these conditions, as compared to the turbidity reversal studies at a temperature of 10 °C, thermal reversibility was seen to be more complete. This property has been observed for type I collagen prepared from lathyritic sources (Gross, 1963) in which it has been found that the lateral assembly, but not the linear assembly, can be thermally reversed (Gelman & Piez, 1980).

Characterization of the EHS Collagen Complex by Velocity Sedimentation. In order to provide a better characterization of type IV collagen before and after complex formation and to analyze for stable intermediates, rate zonal velocity sedimentation was used (Figure 3). For these experiments, the EHS collagen was mixed with trace amounts of ^{125}I-labeled dimeric collagen to increase the sensitivity of the measurements, and the HR9 collagen was used, as before, labeled biosynthetically with [^{35}S]methionine. When centrifuged in the cold in neutral phosphate buffer, type IV dimeric collagen sedimented with an apparent Svedberg value of (5.4 ± 0.2) × 10^{-13} s (n = 10) while the HR9 type IV monomeric collagen sedimented with an apparent value of (4.7 ± 0.1) × 10^{-13} s (n = 5), the latter in fairly close agreement with published data (Duncan et al., 1983). If one assumes a rigid rod configuration (length 424 nm, diameter 1.5 nm) and ignores the

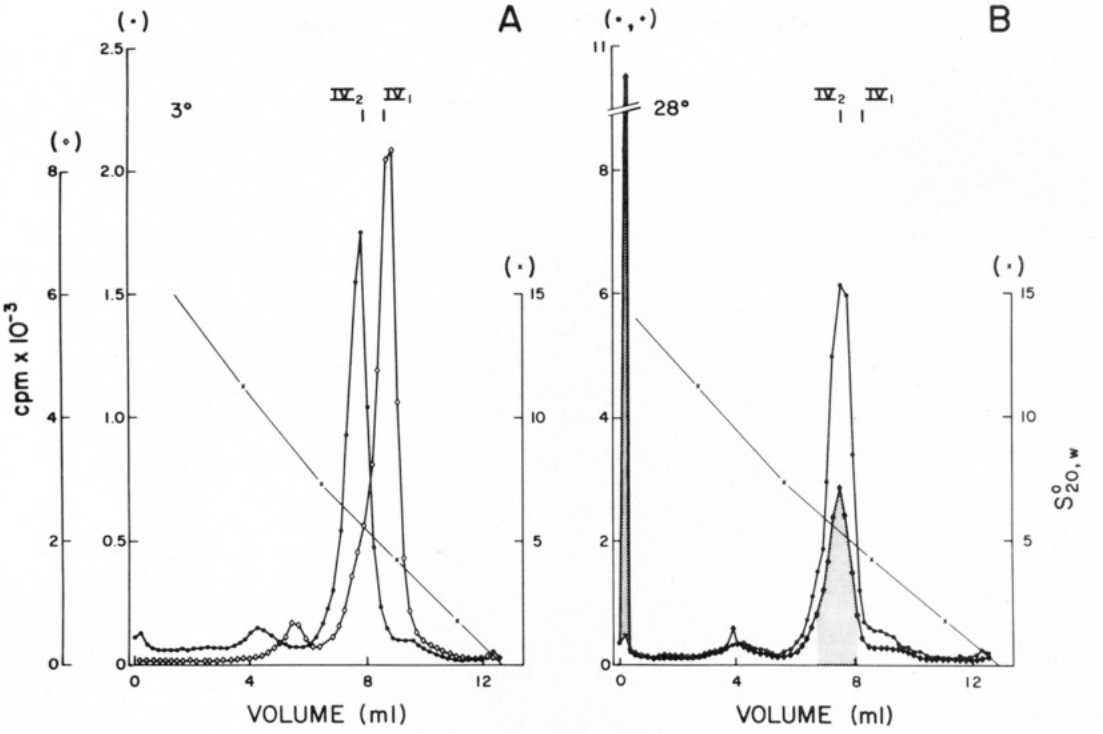


FIGURE 3: Analysis by velocity sedimentation of type IV collagen incubated under various conditions. EHS type IV collagen was trace labeled with ^{125}I-labeled dimeric collagen. After dialysis into neutral phosphate buffer at 4 °C, the preparation was centrifuged at 40 000 rpm for 10–20 min at 3 °C. The supernatant was either maintained at 0–3 °C, incubated at 28 °C for 1 h, or dialyzed into 2 M urea and incubated at 28 °C. PF-HR9 type IV collagen, labeled biosynthetically with [^{35}S]methionine and purified by gel filtration, was used as a monomeric standard. Velocity sedimentation was done at 28 or 3 °C and run with standards in parallel (×) (see text). Average dimeric (IV_2) and monomeric (IV_1) type IV collagen Svedberg positions are indicated. (A) EHS dimeric collagen (closed circles) and HR9-derived monomeric collagen (open circles) maintained in neutral phosphate buffer and sedimented at 3 °C. (B) EHS collagen, at about 0.4 mg/mL dry weight (diamonds, shaded area) and about 0.005 mg/mL (circles), incubated at 28 °C in neutral phosphate buffer for 1 h and sedimented at that temperature.

contribution of the C-terminal globular domain, one can use a theoretical equation (Bloomfield et al., 1967) to estimate the frictional coefficient for monomeric type IV collagen. Assuming a partial specific volume for collagen of 0.71 mL/g, one then predicts an s value for monomeric collagen (M_r 540,000) of 4.3×10^{-13} s, close to the observed value. When the same equation is used, the predicted value for the type I collagen monomer is 3.0×10^{-13} s [observed value is $(2.8–2.9) \times 10^{-13}$ s; Furthmayr et al., 1983]. We suspect that the discrepancy between the predicted and the observed values for type IV collagen is due to the contribution of the globular domain and the increased flexibility of the type IV molecule as compared to type I collagen.

When the EHS collagen was incubated in neutral phosphate buffer at 28 °C at various concentrations and then centrifuged by the rate zonal method at that temperature, a portion of the dimeric collagen now sedimented to the bottom of the tube (greater than 14 S). The amount of collagen recovered at the bottom of the tube depended on the initial concentration (Figure 3B). No stable intermediates between 5.4 and 14 S were identified in these runs. The observation that at different concentrations the ratio of the dimeric to multimeric form changes while stable intermediates do not appear is consistent with a highly cooperative binding process (Engel & Winklmair, 1972).

It has been reported that both glycerol and sucrose inhibit fibrillogenesis of type I collagen (Hayashi et al., 1972). However, the thermal precipitability of type IV collagen does not appear to be affected by sucrose (comparison of direct and sucrose gradient centrifugation data), and type IV collagen will form a turbid mixture in the presence of 50% glycerol (data not shown). Glycerol does appear to have an effect on the extent of lateral assembly as seen by rotary shadowing and as discussed in the next section.

When EHS collagen was first warmed to 28 °C for 60 min or less and then recooled to 0–3 °C and centrifuged on sucrose gradients in the cold, the peak at the bottom of the tube was not seen, and essentially all of the collagen now ran at 5.4 S, consistent with the previously shown property of thermal reversibility. In addition, no conversion to higher s-value forms was seen when EHS collagen was incubated in PBS at 28 °C in the presence of 2 M urea and sedimented in 2 M urea–sucrose gradients at 28 °C. Hence, 2 M urea inhibited thermal precipitation. Under these conditions, no conversion to the monomeric position of 4.4 S was seen, suggesting that 2 M urea does not affect the "head–head" association of the dimeric molecules. In 4 M urea at 28 °C, several peaks are observed, suggestive of denaturation (data not shown).

Characterization of the EHS Collagen Complex by Rotary Shadowing. When EHS collagen was dialyzed into 0.1–0.2 M acetic acid in the cold, centrifuged to remove preformed aggregates, incubated at 28 °C for up to 30 min, and then prepared for metal shadow casting in the presence or absence of glycerol at room temperature, dimeric forms were seen. In addition, there were scattered minimally complexed forms the exact nature of which was difficult to interpret (Figure 4a). However, when EHS collagen was dialyzed into neutral phosphate buffer (pH 7.35), centrifuged in the cold, incubated at 28 °C for 5–10 min at about 0.2–0.4 mg/mL, and then diluted into 0.2 M ammonium acetate (pH 7) to about 15 μg/mL in the absence of glycerol (or alternatively, when the preparation was dialyzed directly into ammonium acetate with brief incubation at 28 °C), a dramatic change was seen with the electron microscope. In over 50% of the fields examined (Figure 4b), a frequently extensive lattice network in the shape of irregular polygons was noted with extensive side by side

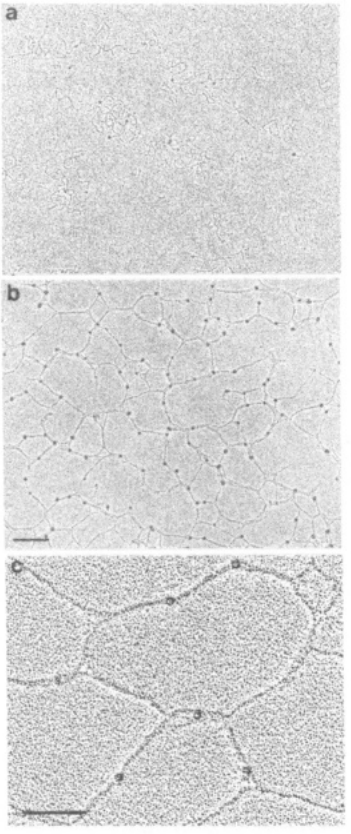


FIGURE 4: Rotary shadow platinum replicas of EHS collagen at neutral and acidic pH. (b and a) EHS type IV collagen was dialyzed into 0.2 M acetic acid or PBS (with 0.1 mM DTT and 0.1 mM EDTA) and then centrifuged at 40 000 rpm for 20 min at 3 °C. The neutral solution, at about 0.4 mg/mL, was incubated at 28 °C for 5 min and then diluted to about 15 μg/mL into 0.2 M ammonium acetate at room temperature. The acid solution was diluted into 0.2 M acetic acid. After 10–15 min, the samples were sprayed onto mica sheets and shadowed with platinum (see text). pH 3 (a); pH 7.5 (b). Over 80% of the fields of sample b were laterally associated. Bar equals 200 nm. (c) EHS type IV collagen was incubated at neutral pH and then diluted into 0.1 M ammonium acetate containing 50% glycerol. An estimated 5–15% of the collagen was laterally associated; the remainder was present as dimers and monomers (about an 8:2 ratio) or occasional scattered smaller complexes such as those shown in Figure 13. Bar equals 100 nm.

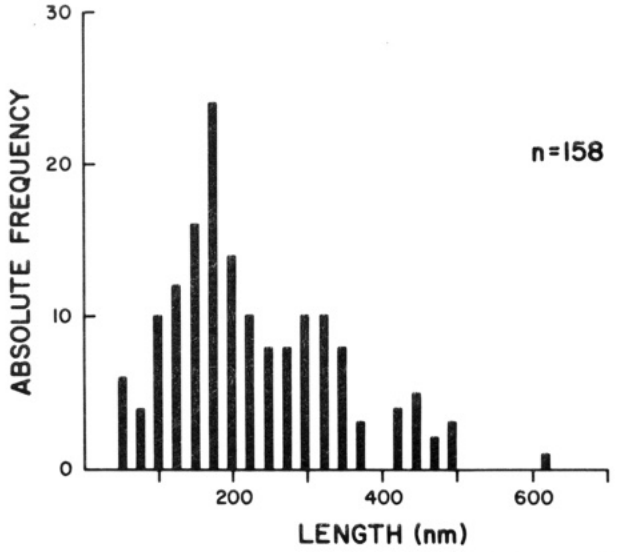


FIGURE 5: Lattice stagger dimension as determined from the distribution of the globular domain of type IV collagen. EHS collagen was shadowed as described in Figure 4c, and the distance between individual globular domains was measured (nearest neighbors) as described in the text.

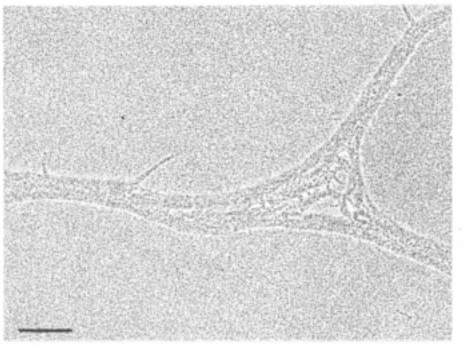


FIGURE 6: Rotary shadow platinum replica of type I collagen. Acid-soluble mouse type I collagen (0.2 mg/mL) in 0.1 M acetic acid was dialyzed into either PBS or 0.2 M acetic acid, warmed to 28 °C for 30 min, and then diluted to 10 μg/mL in acetic acid or 0.2 M ammonium acetate, pH 7.5 (from PBS). After an additional 15–20 min, the samples were sprayed onto mica sheets and shadowed as described. Monomers were seen in acetic acid. Fibers, as shown, were seen at neutral pH.

association of the collagen, two and three triple-helices thick. Thick filamentous bundles were not seen. No apparent difference was found whether purified dimeric type IV collagen was used for the experiment or whether the type IV preparation contained 20% monomeric molecules. The (C-terminal) globular domains were identifiable lying along the sides of these polygons with some 58% residing at the vertices. "7S" tetrameric domains were not seen. Since 7S domains were not identified following a 5–10-min incubation even in those areas which lack extensive lattice formation (less than 10% of the fields in the particular experiment shown), yet could be easily identified following long-term incubation (cf. Figure 8), we concluded 7S complexes were not present in appreciable amounts.

When the collagen was shadowed at neutral pH *in the presence* of 50% glycerol, the number of fields showing the lattice network was reduced to an estimated 5–15% of fields (with an increase in fields showing partial lateral associations), but was otherwise identical in appearance. A high magnification of such a field is shown (Figure 4c). Closer examination of images of this matrix suggests that many of the globular domains were spaced apart by an equal distance from each other. Nearest-neighbor distance measurements were made between individual globular domains in these complexes (Figure 5). A major peak was identified at about 170 nm with a secondary peak at about 310 nm, nearly twice the dimension of the first peak. This 170-nm measurement has been made in the lattice network seen following shadowing in both the absence and presence of glycerol. Hence, there appears to be a median stagger dimension of 170 nm for the dimers participating in the complex.

In contrast to the behavior of type IV collagen, type I collagen showed an entirely different behavior. In acetic acid, type I collagen was seen as monomers and occasionally as small complexes (dimers), but no larger forms could be identified. However, after dialysis in the cold against PBS at neutral pH, centrifugation at 40 000 rpm, and incubation at 28 °C for about 0.5 h, the collagen was seen organized into bundles or even longer fibrils, composed of many molecules, and was unlike the appearance of basement membrane collagen (Figure 6). Branching structures were clearly seen, but no lattice network was identified.

Formation of 7S Associations with Dimeric Type IV Collagen. A second type of association could be studied when EHS type IV collagen dimers were incubated at room tem-

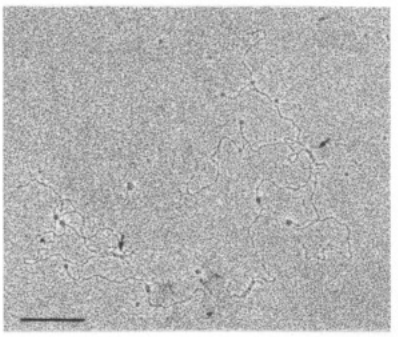


FIGURE 7: Rotary shadowing platinum replica of 7S-bonded EHS collagen. EHS collagen (about 0.4 mg/mL) was dialyzed into PBS, pH 7.9, and incubated at 28 °C for about 20 h. The sample was then diluted to about 10 μg/mL in 0.1 M ammonium acetate, with 50% glycerol, and shadowed. Frequent tetramers, hexamers, octamers, and even larger 7S-bonded complexes were seen as shown in the figure. We estimated that 20% of the free N-termini (36/180) participated in 7S bonding. Some of the large complexes also appeared to be laterally associated and in some of the fields were extensively aggregated (not shown). Bar equals 200 nm.

perature in TBS for 24 h. Rotary shadowing of the preparation in the presence of glycerol (Figure 7) revealed the presence of large numbers of tetramers, hexamers, and octamers which apparently were associated by the 7S domains of the molecules. These structures are reminiscent of the tetrameric spiders isolated from human placenta or the EHS tumor matrix by pepsin digestion (Timpl et al., 1981; Kühn et al., 1981). Because of the predominantly dimeric nature of the starting material, the most completely assembled forms (per 7S domain) constituted octamers, but even larger forms could be observed in which a dimer bridged two 7S domains. In addition to these readily identifiable structures, there were also scattered complexes seen which appear to have assembled by lateral associations within the long collagenous portion of the molecules and also scattered unresolvable aggregates (the latter increased in frequency compared to short-term incubations).

In Vitro Self-Associations of Pepsin-Generated EHS Monomers. In order to determine whether the globular domain or the dimeric nature of type IV collagen per se contributes to the formation of the network shown above and to isolate the N-terminal-end association from lateral associations, EHS collagen was treated with pepsin under gentle conditions. This treatment yielded monomers lacking the globular (C-terminal NC1) domain. On $NaDodSO_4$–acrylamide gels, the $\alpha 1$ and $\alpha 2$ chains were seen to now migrate as a single band at a slightly reduced molecular weight of about 165 000. Rotary shadowing (Figure 8, inset) revealed collagenous strands with a median length of 410 nm for about 80% of the material and less (about 250 nm) for the remainder (Figure 8). These data are consistent with the generation of monomeric type IV collagen by cleavage near the globular domain of the dimeric form. Less than 1% of the molecules still contained the globular C-terminal region.

When the pepsin-treated collagen was subjected to velocity sedimentation at 3 °C in neutral phosphate buffer, an apparent Svedberg value of $(4.4 \pm 0.2) \times 10^{-13}$ s ($n = 16$) was obtained which was slightly less than the previously established value for the intact monomeric molecules (Figure 9A). However, after incubation at 28 °C for 1 h and sedimentation either at 28 °C or at 3 °C, much of the 4.4S collagen was converted to a new peak at $(7.7 \pm 0.3) \times 10^{-13}$ s ($n = 14$) (Figure 9A,B). Up to 1 mg/mL, no significant amount of material was seen to migrate faster than 7.7 S although above that concentration some faster migrating material was seen as well. Thermal

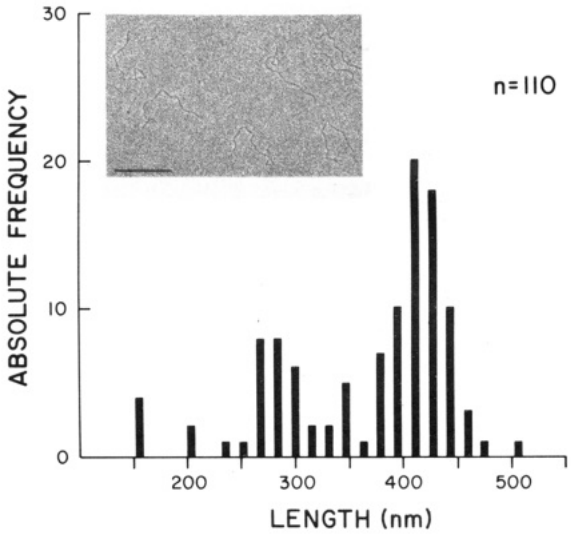


FIGURE 8: Characterization of pepsin-generated monomeric EHS type IV collagen. EHS type IV collagen was treated with pepsin and purified by gel filtration as described in the text. Histogram of pepsin-treated type IV collagen lengths measured from rotary shadow electron micrographs as described in the text. The platinum replica (inset) was obtained by rotary shadowing of pepsin-treated collagen at about 10 μg/mL, in 0.1 M ammonium acetate containing 50% glycerol. Bar equals 200 nm.

reversibility was not observed although incubation in 2 M urea dissociated the 7.7S complex to the size of the original material. The incubated pepsin-treated collagen product could also be separated from dimeric and monomeric collagen by gel filtration (Figure 10) with an estimated molecular weight of 2×10^6, the size of a tetramer. This experiment supported the interpretation that the newly formed peak at 7.7 S corresponded to tetrameric material. Proof for this interpretation was provided by rotary shadowing since the molecules had the appearance of "spiderlike" tetramers (Figure 11). These spiders are essentially identical with those derived by pepsin extraction of basement membranes (Timpl et al., 1981; Kühn et al., 1981) and are similar to those formed in vitro from monomeric type IV collagen (Duncan et al., 1983) except that they lack the globular domains.

The rate of tetramer formation was estimated (Figure 12) by incubating pepsin-treated type IV collagen at different concentrations at 28 °C for increasing periods of time followed by velocity sedimentation at 3 °C to resolve the monomeric and tetrameric peaks. At zero time, a measurable fraction of tetramers was observed that probably indicated the contribution from a very slow rate of conversion at 0–3 °C which occurred either during dialysis or during centrifugation, for we cannot exclude some small degree of equilibration between peaks during the centrifuge run. The graphed lines could be extrapolated to zero. The observed half-time of tetramer formation, for samples containing between 0.1 and 0.4 mg/mL, of approximately 6 h reflects a rate that is about an order of magnitude slower than the rate of turbidity formation for the intact (and mostly dimeric) collagen (0.5-h half-time) at a similar concentration. Long-term (9–20-h) incubations of monomeric collagen at different concentrations (data not shown), conditions at or nearly at equilibrium, demonstrated a concentration dependence for tetramer formation which was also different from that for multimer formation from dimeric collagen.

Discussion

Types of Basement Membrane Collagen Self-Assembly. In this study, we have analyzed three types of noncovalent

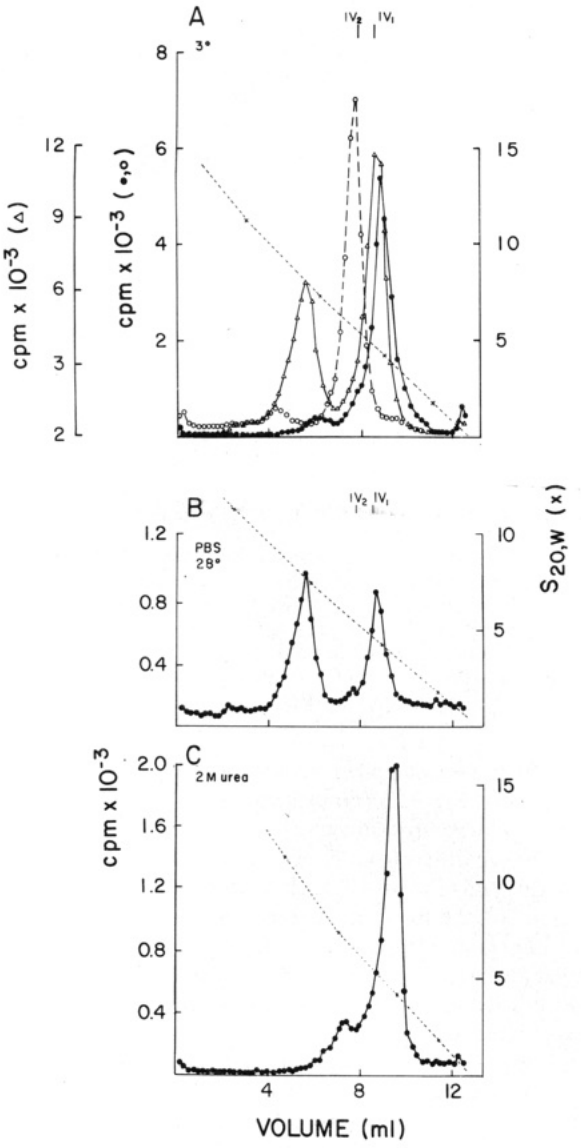


FIGURE 9: Velocity sedimentation of pepsin-treated EHS type IV collagen. (A) Pepsin-treated collagen (0.2 mg/mL) was dialyzed into neutral phosphate buffer in the cold prior to analysis by velocity sedimentation (closed circles): EHS dimeric collagen (0.4 mg/mL) in the same buffer (open circles); pepsin-treated collagen (1 mg/mL) incubated at 28 °C for 1 h (open triangles); protein standards run in parallel tubes (×). (B) Pepsin-treated collagen (0.3 mg/mL) incubated in neutral phosphate buffer for 6 h followed by velocity sedimentation at 28 °C. (C) Pepsin-treated collagen (0.3 mg/mL) incubated in neutral phosphate buffer for 4 h followed by 2 M urea for 2 h.

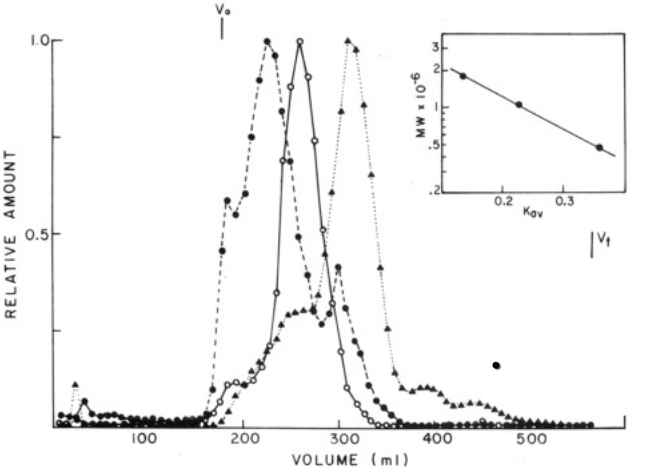


FIGURE 10: Gel filtration of pepsin-treated monomeric, dimeric, and tetrameric EHS type IV collagen derived from pepsin-treated type IV collagen on Sephacryl S1000. ^{125}I-labeled pepsin-generated monomeric collagen (closed triangles), EHS dimeric collagen (open circles), and pepsin tetrameric collagen (closed circles) were chromatographed in neutral phosphate buffer on a 2.5 × 95 cm Sephacryl S1000 column at 4 °C. The tetrameric collagen was formed by incubating pepsin-treated monomers (about 2 mg/mL) at 28 °C overnight prior to chromatography.

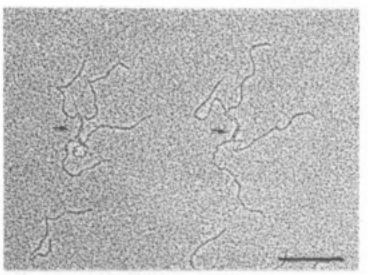


FIGURE 11: Platinum replica of rotary-shadowed EHS–pepsin collagen tetramers. Pepsin-generated monomers (0.8 mg/mL) were incubated in neutral phosphate buffer at 28 °C overnight and then diluted to 10 μg/mL in 0.1 M ammonium acetate with 50% glycerol. The samples were shadowed as described in the text. Lattice complexes were not seen. Bar equals 200 nm.

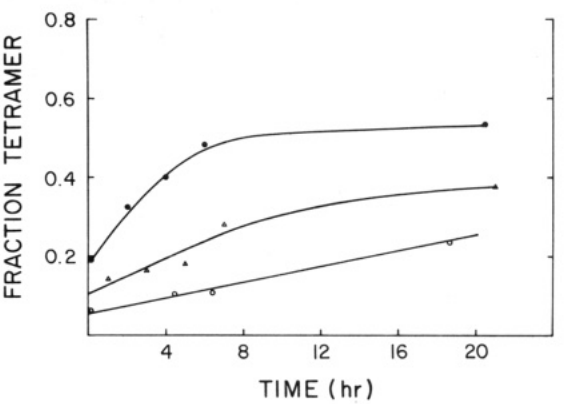
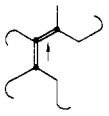


FIGURE 12: Rate of tetramer formation in vitro. Pepsin-generated EHS monomeric collagen (4 mg/mL) was diluted into neutral phosphate buffer to 0.37 (closed circles), 0.12 (open triangles), and 0.09 mg/mL (open circles) and then further dialyzed at 0 °C for 1 h just prior to warming; 0.4-mL samples were incubated at 28 °C for various times, and incubations were terminated by placing the samples on ice followed immediately by velocity sedimentation at 3 °C. The ratio between tetrameric and monomeric radioactivity was compared and expressed as the fraction of tetramer formed.

self-associations of basement membrane collagen. A summary is presented in Table I. It was possible to demonstrate in vitro self-assembly for two of these types.

So far, it has not been possible to manipulate the first of the three associations, the C-terminal globular or head–head interaction. The dimeric associated form of type IV collagen is already preexistent when the tumor matrix or the matrix deposited in tissue culture is extracted. We have been unable to interconvert dimers and monomers in vitro: preliminary experiments indicate that while monomers isolated from the medium of HR9 cells will form tetramers by association within the N-terminal 7S domain, they will not form dimers via the globular C-terminal region (although we cannot rule out the latter conversion at higher concentrations). We doubt that our failure to dissociate dimers into monomers is due to the presence of covalent bonds since the collagenase-generated dimeric C-terminal globular domain will dissociate at least to a large extent into individual 25 000-dalton polypeptide fragments in 2 M urea (Aristides Charonis, Yale University School of Medicine, personal communication).

Table I: Types of Interactions in Self-Assembly of Type IV Collagen

bond	location	product	region	thermal reversibility	rate	dissociation conditions[a]
"head-head" (NC1) 	C terminal	dimer (end to end)	noncollagenous	no	?	resistant to 2 M urea + DTT, 28 °C
"7S" 	N terminal	tetramer (end overlap)	collagenous	no	slow	2 M urea + DTT
collagenous 	length of chain	multimer (lateral, staggered)	collagenous	yes	fast	2 M urea; cold; acetic acid

[a] In lathyritic material.

The second type of association listed in Table I is the end-region 7S bond. Dimeric or monomeric intact type IV collagen and pepsin-generated monomeric type IV collagen can self-assemble in vitro to form high molecular weight tetrameric structures which are associated within a 30-nm-long N-terminal segment of the molecule. Aspects of this process have been reported for monomeric collagen derived from the PF-HR9 cell line (Duncan et al., 1983) in which it has been shown that both noncovalent and disulfide bonding contribute to 7S formation in vitro. In our system, we find that the conversion can proceed in the absence of interchain disulfide formation (reversible in 2 M urea) and that the specificity of bond formation therefore lies in noncovalent associations. This process appears to be quite different from that leading to lattice formation. While, like the lateral associations, tetramer formation is inhibited and reversed by 2 M urea in the presence of reducing agents, the rate is substantially (about 1 order of magnitude) slower as compared to multimer formation, and the reaction is not thermally reversible. This is evident from studies on pepsin-treated type IV collagen which allowed determination of the amount of the tetrameric product and which also allowed distinction between the higher molecular weight tetramer from the even larger multimeric lattice product. Since long-term incubation is required at lower concentrations to generate 7S complexes in vitro, and since tetramer formation is resistant to thermal reversal, we conclude that the majority of lateral bonds form prior to significant N-terminal 7S formation. This hypothesis is supported by the extent of thermal reversibility of intact collagen complex formation (at less than 0.2 mg/mL) up to at least 4 h, and it is also supported by our inability to demonstrate by rotary shadowing significant numbers of type IV collagen spiders at early incubation periods. It appears that both associations are formed in the long-term incubation experiments with the dimeric preparation and that glycerol decreases (but does not entirely eliminate) lateral associations while preserving the 7S complex. Although tetramers can form at a relatively slow rate in the absence of extensive lattice formation, we have not been able to directly measure the rate of formation of the 7S domain in the presence of side by side associations. However, one might expect an even slower rate of 7S bonding under these conditions since lateral associations should decrease the translational and rotational energy of the system and thereby reduce the rate of molecular collision.

The third type of association listed in Table I is that of lateral assembly, distinct from end associations. Dimeric type IV collagen assembles into large complexes of increased viscosity in PBS or other salts following warming to 28 °C. This complex, at least initially, is capable of dissociating back into dimers in the cold. The collagen used in this study was prepared from lathyritic tumor and hence is largely incapable of forming subsequent hydroxylysine-dependent covalent cross-links. The process of polymerization of lathyritic type I collagen is also thermally reversible (Gross, 1963). The aggregation process is inhibited completely by 2 M urea and will not proceed at pH 3 in acetic acid. The latter quality is shared by type I collagen undergoing fibrillogenesis in vitro. Type I collagen, however, shows an initial lag period, representing the formation of linear trimers (Silver, 1979; Trelstad & Silver, 1981). This lag period has not been appreciated for the EHS-derived type IV collagen. The rate of turbidity increase, presumably reflecting the rate of complex formation, is fairly rapid with a half-time of formation at 0.2 mg/mL of about 35 min. Viscosity increases at a much slower rate and may reflect the formation of both lateral and end associations. Complex formation is found to be a concentration-dependent process with no identifiable stable intermediates between 5.4 and 14 S, suggestive of a highly cooperative binding process. The rotary shadowing method provided an important tool by allowing visualization and further analysis of the formed complex. A latticelike network of irregular polygons, formed by side by side association of apparently collagenous domains generally two to three triple-helices thick, is seen. Large filamentous bundles are not observed in contrast to type I collagen. The C-terminal globular domains can be clearly identified and frequently (58%) reside on the polygonal vertices. These globular "head" structures serve as orientation markers for each molecule and are spaced apart by a median distance of 170 nm. We interpret this as representing a stagger dimension equal to one-fifth the length of a dimer (170/840 nm). Type I collagen, on the other hand, forms fibers with 67-nm stagger. Careful measurements of the major flexible loci ("kinks") of type IV collagen (Hofmann et al., 1984) reveal that they are spaced apart by about one-fifth the length of a monomer, dividing the monomer into five collagenous domains of about equal length (the N-terminal domains contain more extensive flexibility). The 170-nm stagger dimension therefore appears to correspond to the length of two such domains. If one allows one C-terminal head to lie on one such flexible locus, the stagger would determine that successive heads in the complex will lie on every other available kink.

We thus appear to have identified a novel type of collagenous association. Although we cannot entirely exclude the possibility that the noncollagenous subdomains within the major triple-helical domain actually are responsible for the formation of the complexes, it would seem more likely by

analogy to the interstitial collagens that the collagenous triple helix in fact participates in this binding.

It is not clear why intact monomeric collagen (at least at lower concentrations) and pepsin-treated collagen (below 1 mg/mL) do not appear to form identifiable lateral associations. While the C-terminal globular domain may be important for initiation of lateral complex formation in a manner analogous to the telopeptides for initiation of type I lateral assembly, it would also appear that the dimeric configuration, perhaps due to its amino- to carboxy-terminal antivectorial arrangement, is also required for lateral assembly.

Duncan et al. (1983) have shown that 7S tetrameric collagen can be generated in vitro from the monomeric type IV collagen isolated from the medium of PF-HR9 cells (Bächinger et al., 1982). These data could be interpreted to indicate that 7S tetramer formation constitutes the initial step in the assembly of this collagen. However, there are several arguments against this interpretation. First, if one examines the matrix deposited by these cells, the predominant form of collagen found is dimeric (unpublished results) similar to the EHS tumor matrix. Second, monomeric collagen molecules from the PF-HR9 cell medium are not seen to form dimers in vitro (Duncan et al., 1983; P. D. Yurchenco, unpublished results). Third, 7S tetrameric forms generated from monomeric collagen do not appear to polymerize to larger structures (Duncan et al., 1983). Thus, it is not apparent from these data how a network would be established. More systematic studies with monomeric collagen assembly, particularly with respect to dimerization, are required to settle this issue. However, this will require larger amounts of monomeric collagen than we have been able to prepare to date.

Speculations on the Matrix Assembly of Type IV Collagen. The earlier rectilinear matrix model, based upon end associations only (Timpl et al., 1981), should now be modified to accommodate lateral associations as well. Such a matrix model would provide a more rigid and tighter matrix. Obviously, this model and also the alternatives we have considered do not take into consideration contributions to the self-assembly process of type IV collagen by molecules such as laminin, entactin, heparan sulfate proteoglycan, and other molecules synthesized by cells and present within basement membranes, nor do they take into consideration cellular control mechanisms which may exist in vivo. Furthermore, we do not yet know whether the lateral associations become subsequently covalently cross-linked with resultant stabilization in nonlathyritically derived collagen.

With these limitations in mind, how might such a type IV collagen matrix be assembled? One sequence is suggested by the series of rotary shadow images shown in Figure 13. Free dimers would begin to laterally self-associate to form side by side tetramers, distinct from the end-region 7S tetramers. Additional dimers would then laterally associate to form hexamers and larger forms with eventual completion of a lattice matrix. Let us consider the possibility that what is seen in vitro by rotary shadowing is an imperfect representation of the matrix of an actual basement membrane, not only because of the technique itself which involves compression of a three-dimensional structure into two dimensions but also because the conditions of assembly underneath a secretory cell forming a basement membrane are not identical with the conditions in vitro. In particular, we would like to propose the possibility that what forms, or can form, in vivo, is a *regular* polygonal matrix.

In the consideration of geometric alternatives, several restrictions, on the basis of the observed data, were imposed.

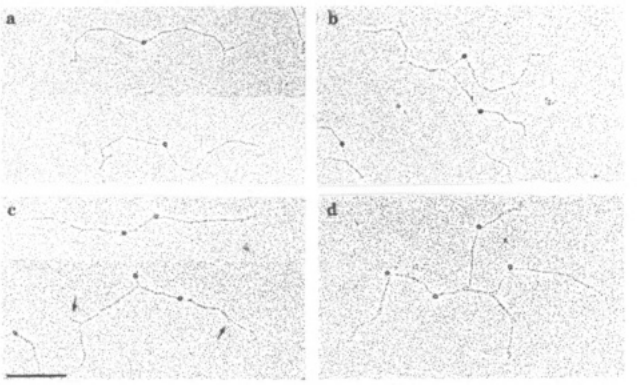


FIGURE 13: Platinum replicas of rotary-shadowed type IV collagen intermediate complexes. These highly selected micrographs suggest a mode for early assembly in which free dimers (a) laterally associate (b) in stagger to produce unstable side by side tetramers (c), distinct from the 7S tetramers. Additional dimers participate in binding (d) with eventual formation of a matrix. Bar equals 200 nm.

First, the dimer was considered to be the most likely basic building block for the matrix. In the experiments described, it was the dimeric form which was seen to form the multimers. Second, lateral binding was allowed to occur with a one-fifth (170 nm) dimer length stagger. The actual rotary shadow measurements indicated that the globular domains, located in the center of a dimer, are spaced predominantly by this distance, and one can interpret the observed data as a statistical averaging process reflecting a single stagger dimension. The third restriction was that all globular domains reside on the vertices of whatever geometric pattern was tested and was an extrapolation of the observation that 58% were found on the vertices (which is much greater than would be expected purely in a random distribution). This raises the possibility that the heads may act as branch-point signals for lateral assembly. The second and third rules and the previously discussed one-tenth dimer length kinks (Hofmann et al., 1984) result in half of the major flexible loci of the collagen lying directly on the vertices of the geometric array. Fourth, triple-helical strand thickness was limited to two or three since one rarely saw matrices that exceeded that number. The fifth and last restriction was that the ratio of dimeric heads to polygonal side to N-termini must be 1:4:2 in the tessellating geometric unit. This was simply the consequence of the length of a dimer (840 nm), the stagger length (170 nm), and the C-terminally coupled state of the dimer. Using these restrictions, one finds that there are few geometric possibilities, particularly if one limits the models to layered arrays, as suggested by morphological studies of basement membranes (Carlson et al., 1981; Sawada, 1982).

What we consider to be the best model and its proposed assembly are shown in Figure 14. It is a layered regular hexagonal planar array whose sides measure 170 nm (stagger dimension), whose vertices are formed by 120° angles, and whose planes, held together by 7S bonds, are spaced by 140 nm. In the suggested sequence of assembly events, free dimers laterally associate with a one-fifth length stagger to form tetramers, then hexamers, and then dodecamer hexagons. The free arms of the hexagons laterally associate to form a hexagonal array. This results in free N-terminal pairs at each vertex, each 85 nm long, which are in exact register to participate in 7S bond formation with a second series of free paired N-termini (Figure 14B) located on the vertices of a second hexagonal plane. The two planes could be superimposed with each hexagon directly over the next (resulting in all N-termini involved in 7S bonding) or could be offset to

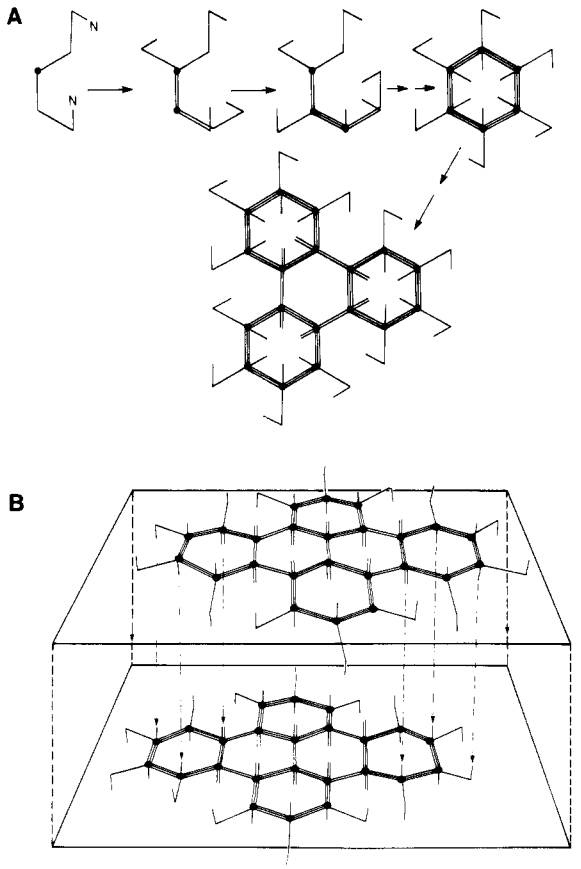


FIGURE 14: Suggested mode of type IV collagen assembly with formation of a layered regular hexagonal array. (A) Dimers laterally associate, as suggested in Figure 13, to form staggered tetramers and then larger side by side complexes with formation of dodecameric hexagons. The free arms of the hexagons laterally associate to produce a planar hexagonal array. Note the production of free paired N-termini at each vertex. (B) The free N-terminal pairs are now in exact register to participate in 7S bond formation between two or more planes of hexagons.

allow only half of the N-termini to bind between each plane, permitting an infinite number of possible planes. One of the attractive features of this model is that it places the N-termini in proper register for 7S formation; otherwise, one might expect the 7S bond to form with difficulty due to the lack of mobility of partially bonded dimers with resultant reduced molecular collision frequently between end regions. A second possible ordered model which obeys the imposed restrictions is that of a series of layered planes each composed of an equilateral triangle array (yielding hexagons). All sides (170 nm) are two triple-helical strands thick, and the vertices are formed by 60° angles. However, now two-thirds of the vertices are occupied by paired dimeric globular domains, and the remaining one-third of the vertices are occupied by six pairs of N-terminal domains, again available for 7S binding out of the plane of the array. We consider this model somewhat less likely because we rarely see more than one dimeric globular domain per vertex.

Although the data presented do not allow one to choose between an irregular and a regular lattice network for basement membranes, there is published morphological data to suggest that such hexagonal lattices exist in basement membranes. Descemet's membrane, an unusually thick membrane located underneath the corneal endothelium, has been shown by the deep-etch replica method to be composed of stacks of two-dimensionally arranged hexagonal lattices (Sawada, 1982) whose vertices are spaced apart by an estimated 200 nm. While the rodlike structures which connect the vertices are collagenase sensitive, the nodes at the vertices are not. In cross section, one sees 200-nm spaced bars instead. However, while Descemet's membrane contains type IV collagen (Bornstein & Sage, 1980), it appears to also contain type II collagen (Hendrix et al., 1982) and possibly other collagens, and it is not entirely clear if the type IV collagen makes up the hexagonal array. Renal tubular basement membrane, a more "typical" basement membrane, also has a recognizable regular hexagonal array (Carlson et al., 1981) when viewed "en face" following treatment with trypsin. However, this protease-resistant array has not been proven to be type IV collagen. Therefore, while there are published data to suggest that basement membrane collagen can form hexagonal layered arrays, it has not been firmly established.

In conclusion, lateral as well as C- and N-terminal noncovalent binding participates in the supramolecular organization of type IV collagen. However, the exact organization of this matrix in basement membranes remains to be elucidated and will undoubtedly require a combination of further in vitro reconstruction and in vivo analytic studies.

Acknowledgments

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Identification of Hydroxypyridinium Cross-Linking Sites in Type II Collagen of Bovine Articular Cartilage†

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ABSTRACT: In mature cartilage, collagen fibrils are strengthened by covalent intermolecular bonds provided by 3-hydroxypyridinium cross-linking residues. To determine the location of these trifunctional cross-links within the type II collagen molecule, CNBr peptides were analyzed from pepsin-soluble collagen and from guanidine hydrochloride insoluble collagen of bovine articular cartilage. The presence of hydroxypyridinium residues in collagen α chains and CNBr-derived peptides was detected by their characteristic natural fluorescence. Quantitatively, about one in three α chains from pepsin-soluble collagen was found to contain a hydroxypyridinium residue. Its distribution in the chains was limited to two CNBr peptides, which were purified by column chromatography on CM-cellulose and Bio-Gel P-30 followed by slab-gel electrophoresis in sodium dodecyl sulfate–polyacrylamide. The composition and properties of the two peptides indicated that the main component of one was α1(II)-CB9,7 and of the other α1(II)CB12. It was suspected that two amino-terminal telopeptides were cross-linked by hydroxylysylpyridinoline to α1(II)CB9,7 and two carboxy-terminal telopeptides to α1(II)CB12. The properties of fluorescent CNBr peptides isolated from digests of insoluble cartilage collagen supported this conclusion. Cleavage of the 3-hydroxypyridinium ring by UV light was exploited to confirm the identity of the cross-linked peptides. On UV irradiation, one cross-linked peptide released α1(II)CB9,7, and the other, α1(II)CB12. The findings indicate there are only two hydroxypyridinium cross-linking sites within the triple-helical region of the type II collagen molecule, probably placed symmetrically at opposite ends at residues 87 and 930, where telopeptide aldehydes are known to react to form the initial "head to tail" intermolecular bonds.

A naturally fluorescent amino acid, that embodies three hydroxylysine residues in a 3-hydroxypyridinium ring, was found in acid hydrolysates of bovine achilles tendon and proposed to be a trivalent cross-linking residue of collagen (Fujimoto et al., 1977, 1978). The cross-link, which was named pyridinoline, has since been found in the collagen of most connective tissues other than skin (Fujimoto & Moriguchi, 1978; Moriguchi & Fujimoto, 1978; Eyre & Oguchi, 1980; Kuboki et al., 1981; Eyre et al., 1984) and is especially abundant in articular cartilage (Eyre & Oguchi, 1980; Eyre et al., 1981). During cartilage growth, the divalent reducible cross-link hydroxylysino-5-ketonorleucine predominates (Robins & Bailey, 1974; Parsons & Glimcher, 1976; Shapiro et al., 1979), but in the mature tissue it is almost totally replaced by hydroxypyridinium residues (Eyre et al., 1981). The reducible cross-links are thought to be direct precursors of the hydroxypyridinium cross-links, and there is evidence that two reducibles give rise to one hydroxypyridinium (Eyre, 1980; Eyre et al., 1981).

Two forms of the cross-link have been identified, a major one which embodies three hydroxylysine residues [hydroxylysylpyridinoline (HP)[1]] and a minor one which embodies two hydroxylysines and one lysine [lysylpyridinoline (LP)] (Eyre,

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[1] Abbreviations: HP, hydroxylysylpyridinoline; LP, lysylpyridinoline; CNBr, cyanogen bromide; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Gdn·HCl, guanidine hydrochloride.

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