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Electron-microscopical approach to a structural model of intima collagen

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Intima collagen was studied by electron microscopy (rotary shadowing and negative staining) and by analytical ultracentrifugation. It was found that the monomeric unit (M_r 170000) consists of a 105 nm-long triple helix terminated by a small globular domain (M_r about 30000) at one end and a large globular domain (M_r about 40000) at the other end. The monomer was produced by selective reduction of interchain disulphide bridges. Before reduction, dimers, tetramers and larger filamentous structures were found. Dimers are lateral staggered aggregates of two monomers aligned in an anti-parallel fashion. This gives rise to an inner 75 nm-long region of two slightly intertwisted triple helices flanked by the large globular domains. The outer triple-helical segments (length 30 nm) with the small globular domains at their ends emerge at both sides of this structure. Interchain disulphide bridges are probably located in the vicinity of the large domains. Only the outer segments could be degraded by bacterial collagenase. In tetramers the outer segments of two dimers are covalently linked, forming a scissors-like structure. In the fibrous forms several tetramers are assembled end-to-end with an overlap between the outer segments. The molecular masses and sedimentation coefficients were calculated for these various forms from the electron-microscopically observed dimensions and agreed with results obtained by ultracentrifugation. The unique structure of intima collagen suggests that it originates from a microfibrillar component and that it can be considered a unique collagenous protein, for which we propose the designation type VI collagen.

It was shown in the accompanying paper (Odermatt *et al.*, 1983) and in previous studies (Furuto & Miller, 1980; Jander *et al.*, 1981) that intima collagen is composed of short polypeptide chains (M_r 40000–70000) comprising collagenous and non-collagenous segments. Most probably the basic structural unit (M_r about 160000) consists of a triple-helical segment and two globular domains (Odermatt *et al.*, 1983). Disulphide bonds were found to be important for the stabilization of the monomers and for the interconnection of protomers to higher structures.

Electron microscopy, and in particular the rotary-shadowing technique, was instrumental in demonstrating that basement-membrane (type IV) collagen is organized as a regular network structure (Timpl *et al.*, 1981). The technique has been also useful in analysing the domain organization and assembly of other thread-like proteins, such as spectrin (Shotton *et al.*, 1979; Tyler *et al.*, 1980*b*; Morrow & Marchesi, 1981), laminin, fibronectin (Engel *et al.*, 1981; Erickson *et al.*, 1981), actin-binding protein, filamin (Tyler *et al.*, 1980*a*) and myosin (Lowey *et al.*, 1969; Elliott & Offer, 1978).

In the present study we have analysed intima collagen and fragments derived from it (Odermatt *et al.*, 1983), by electron microscopy after rotary shadowing and negative staining. The results allowed us to propose a model for the assembly of the monomeric units to dimers, tetramers and filamentous structures.

Materials and methods

Isolation and purification of collagens and procollagens

Intima collagen and its fragments were prepared from human placenta as described by Odermatt *et al.* (1983). The materials used in the present study included the two forms A and B of intima collagen, a

fragment Col obtained by digestion with bacterial collagenase and various proteins modified by limited reduction under non-dissociating conditions. A modified form of procollagen III (pN-collagen) was purified from foetal-calf skin (Timpl *et al.*, 1975).

Electron microscopy

Individual molecules were made detectable for electron microscopy by using the rotary-shadowing technique, which was adapted from Shotton *et al.* (1979). Protein samples were dissolved in 50mM-acetic acid (20–50μg/ml), and after addition of an equal volume of glycerol the solution was sprayed on to freshly cleaved mica discs from a distance of 30cm. The samples were then analysed as described previously (Engel *et al.*, 1981; Timpl *et al.*, 1981), or alternatively they were brought into a vacuum chamber of an Edwards vacuum coater model 306 and evacuated to 0.1mPa (1×10^{-6} Torr). An Edwards electron source was used for shadowing the proteins with platinum at an angle of 9°, followed by carbon coating at 90°. A 5cm length of a platinum wire (diameter 0.2mm) was coiled around a tungsten rod of 2mm diameter and completely evaporated at 4kV and an emission current of 50mA. The distance to the mica discs mounted on a rotating table (120rev./min) was 15cm. Carbon was evaporated at 4kV and an emission current between 60 and 100mA was used for 10s. The replicas were cut into 2–3mm-diameter fragments, floated on to distilled water and picked up on 400-mesh copper grids. Specimens were examined in a Siemens Elmiscope 102 electron microscope at 100kV with a 50μm objective aperture. The exact magnification was determined from standards of collagen I (length 297nm) photographed under the same electron-optical conditions.

For negative staining solutions of the proteins at concentrations of 10–30μg/ml in 0.2M-ammonium bicarbonate were applied to thin carbon films that had been rendered hydrophilic by glow discharge. Grids were washed with distilled water and stained with aq. 2% (w/v) uranyl acetate, pH4.1. Specimens were examined in a Zeiss EM 109 electron microscope operating at 80kV with 200μm condenser aperture and 30μm objective aperture.

Analytical ultracentrifugation

Ultracentrifugal measurements in 0.2M-ammonium bicarbonate buffer, pH7.4, were performed at 20°C with a Spinco model E ultracentrifuge (Beckman Instruments) equipped with a photoelectric scanner. Sedimentation-velocity runs were performed at 56000 rev./min, and equilibrium experiments were done in double-sector cells with 0.8–1mm column heights. Molecular weights were evaluated from plots of $\ln A$ versus x^2, where A is the absorbance at 280nm and x is the distance from the rotor centre. A partial specific volume of 0.73cm^3/g was used.

Results

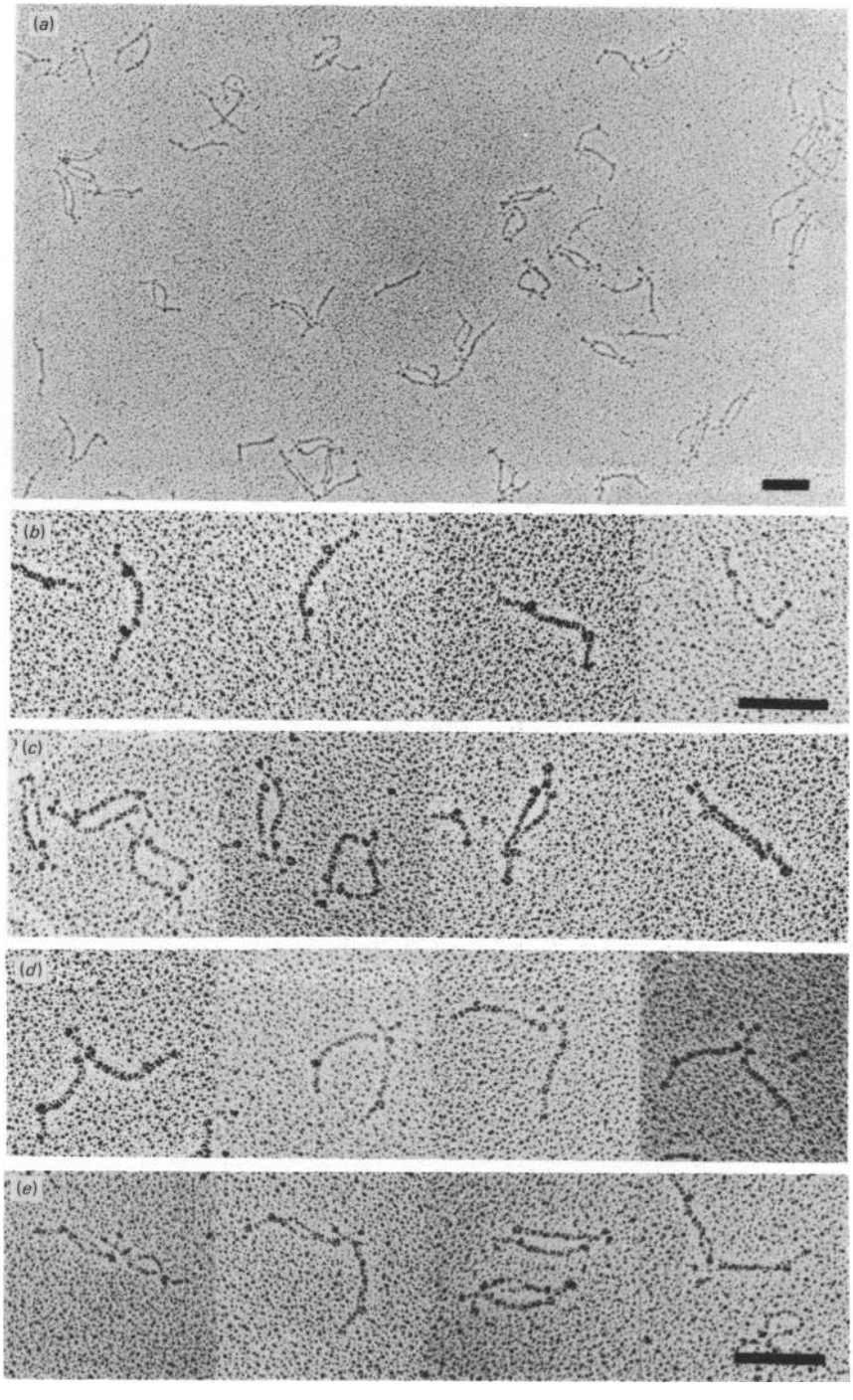
The molecular shapes of intima collagen and its fragments can be determined by electron microscopy. We have used two methods that complement each other, namely rotary shadowing (Plate 1) and negative staining (Plate 2). The first method provides high contrast and is therefore well suited for the determination of thin elongated structures. When negatively stained, such structures are more difficult to trace along their entire length because of low contrast and varying stain thickness. On the other hand morphological details such as globular domains are better resolved by negative staining. Negatively stained particles appear with approximately correct diameters, whereas the decorating effect in rotary shadowing enlarges all dimensions by about 2nm (Elliott & Offer, 1978).

Intima collagen

Intima collagen was obtained by proteolytic digestion of placenta in two forms, A and B (Odermatt *et al.*, 1983). In fields of intima collagen A subjected to rotary shadowing (Plate 1*a*) an abundant species is a symmetrical particle with an inner thick rod-like region (75nm long) with prominent globular structures at both sides. From the inner globular units emerge shorter (30nm) and thinner rod-like segments that are terminated by a less prominent outer globular structure (Plate 1*b*). These particles are designated dimers. It is shown below that they consist of two probably identical triple helices that contain globular domains at both sides.

Dimers are often connected to tetramers by their outer rod-like segments (Plates 1*c* and 1*d*). Connections occur at both ends (Plate 1*c*) or at one end only (Plate 1*d*). In both cases the outer rods of two dimers intersect and form a characteristic scissors-like structure. The point of intersection is about halfway between the inner and the outer globular units. Only rarely are direct contacts observed between the larger globular domains of the two dimers that form the tetramer. An important detail is the occasional but clear separation of two strands in the inner rod-like region, which occurs in both dimers and tetramers (Plate 1*e*). The strand separation indicates that the inner rod consists of two triple helices.

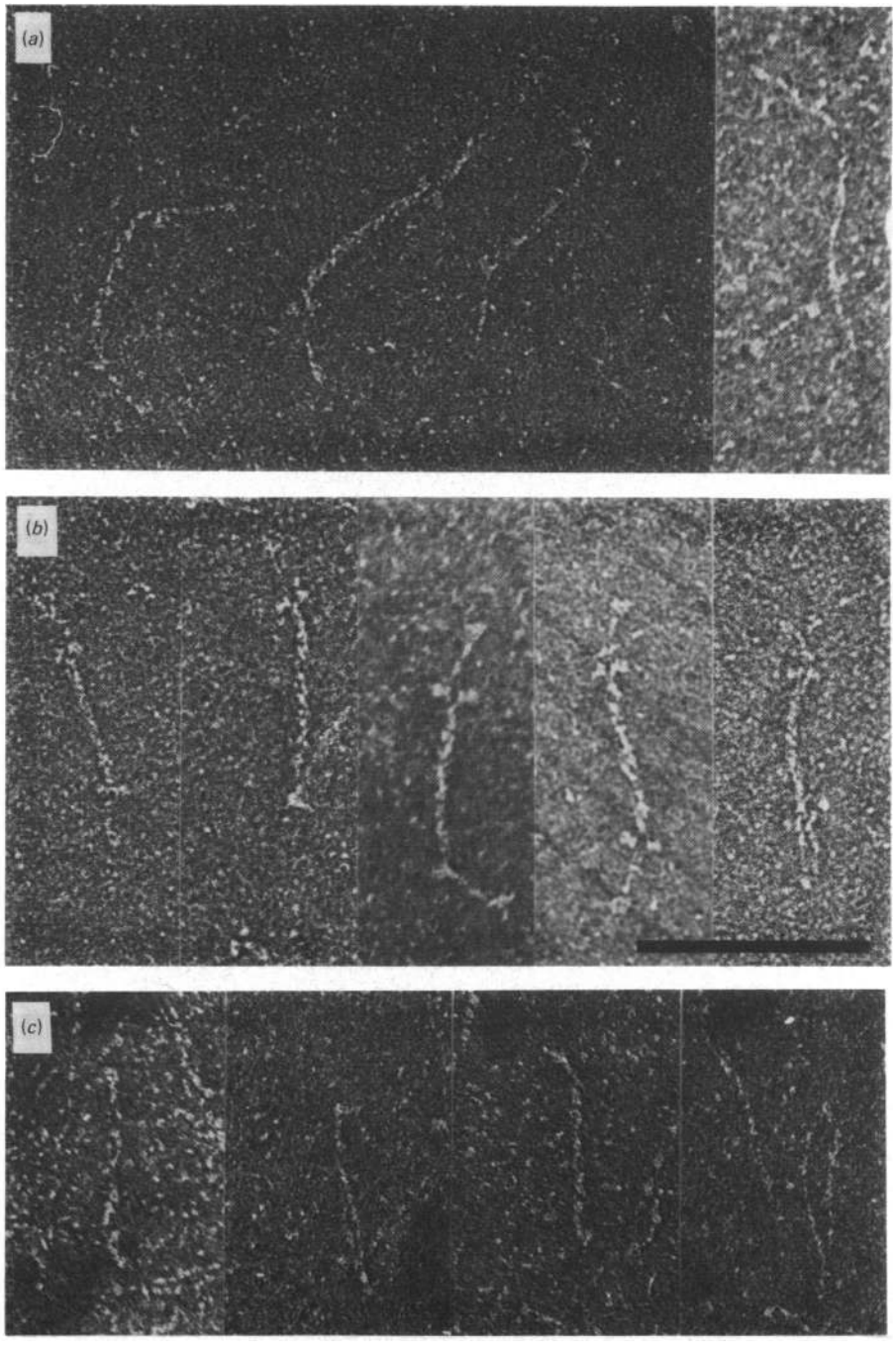
By negative staining the general shapes of dimers and tetramers are confirmed and additional features are revealed (Plate 2). The inner globular units appear as spheres or ellipsoids that are attached at their circumference to the interface between the thick and the thin rod-like regions. There is one domain per interface, and in most cases the protuberances at



EXPLANATION OF PLATE 1

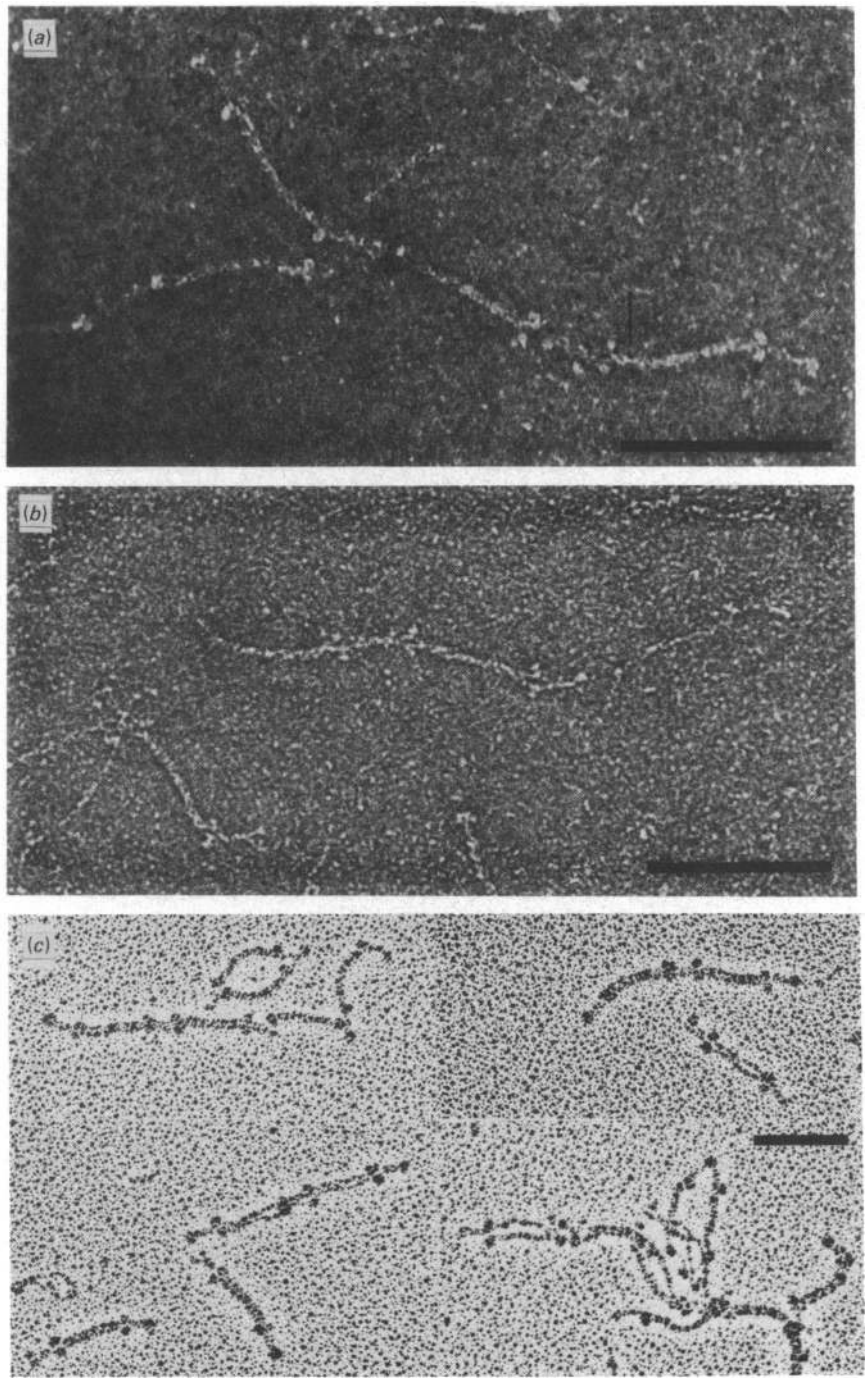
Electron micrographs of intima collagen form A obtained by the rotary-shadowing technique

(*a*) Field at low magnification. Selected particles at higher magnification: (*b*) dimers; tetramers with connections (*c*) at both ends and (*d*) at one end only. (*e*) Particles in which the inner part with two triple helices is partially untwisted. The bars represent 100 nm.



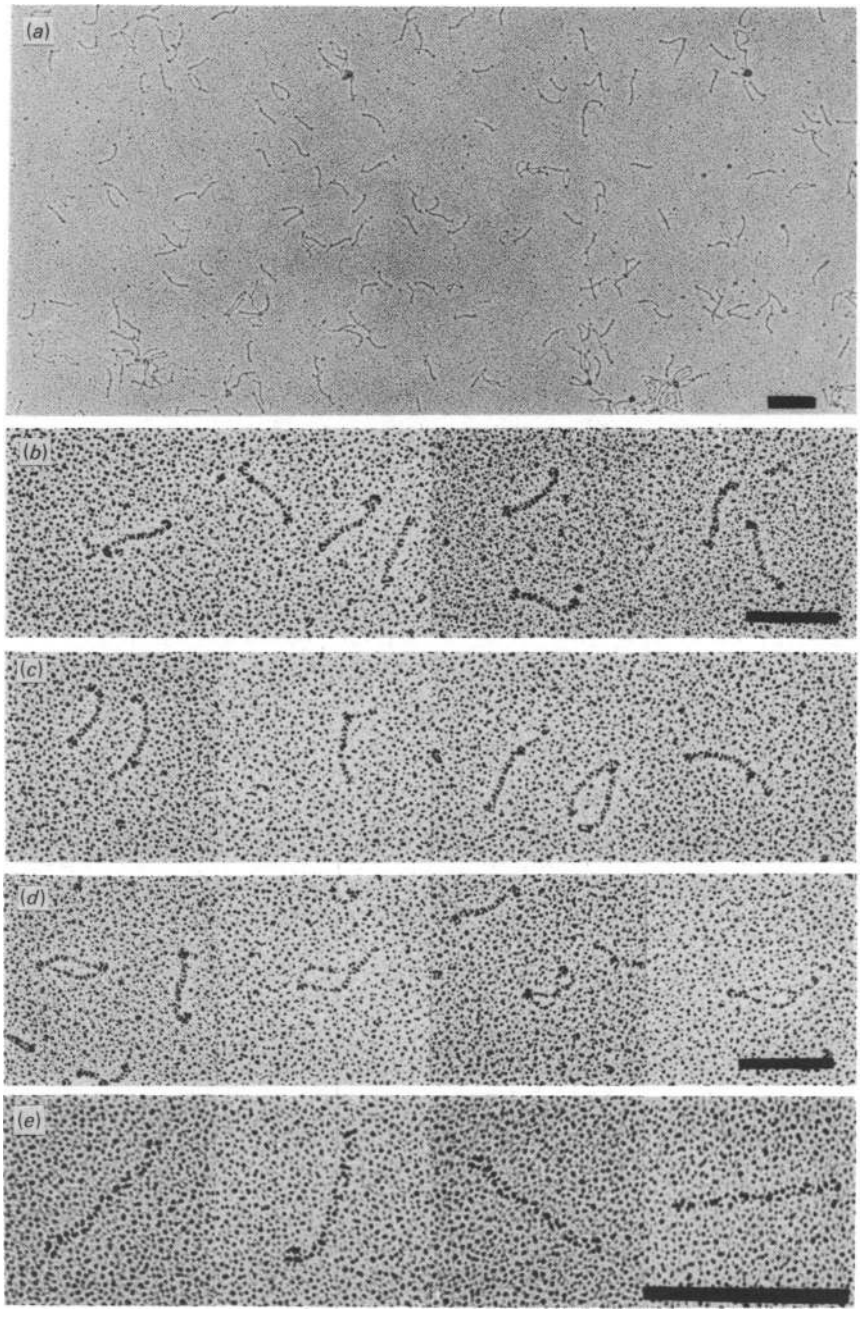
EXPLANATION OF PLATE 2

Electron micrographs of negatively stained intima collagen (*a and b*) *and Col fragment* (*c*)
The upper row (*a*) shows from left to right a dimer, a tetramer and a dimer in a field and a selected dimer. In row (*b*) selected tetramers are shown, and row (*c*) exhibits selected Col fragments. The bar represents 100nm.



EXPLANATION OF PLATE 3

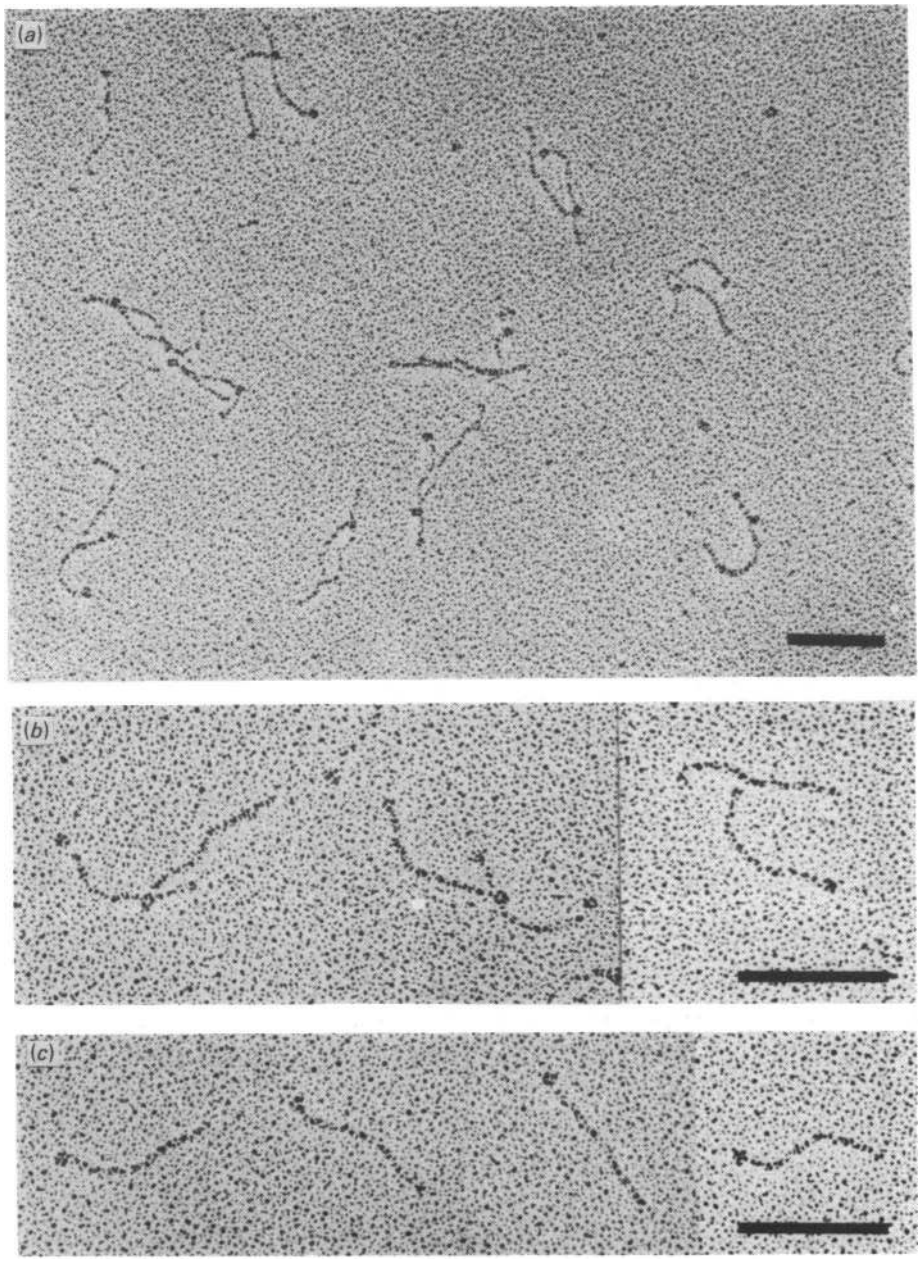
Electron micrographs of fibrous aggregates of intima collagen form A by negative staining (a and b) and by rotary shadowing (c)
The bars represent 100 nm.



EXPLANATION OF PLATE 4

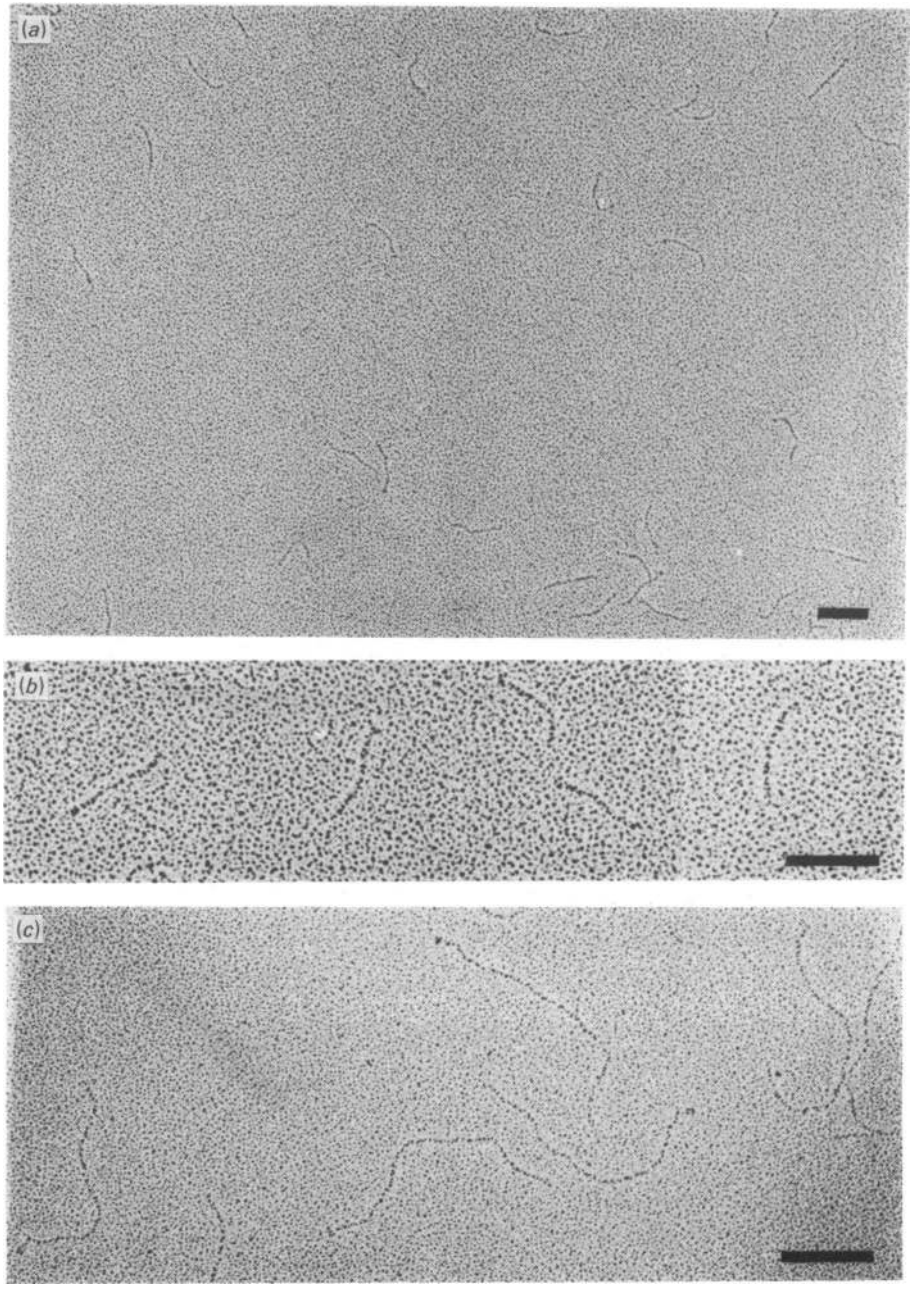
Electron micrographs obtained by the rotary-shadowing technique of fragments of intima collagen form A produced by collagenase digestion

(*a*) Field showing the distribution of particles at low magnification. (*b*)–(*d*) Selected particles at higher magnification with both outer rod-like segments removed (*b*), with outer segments removed from one end only (*c*) and particles in which the two strands between the inner globular units are untwisted (*d*). (*e*) The twisting of the two triple helices around each other shown for particles at high magnification. The bars represent 100 nm.



EXPLANATION OF PLATE 5

Electron micrographs (rotary shadowing) of very mildly reduced intima collagen (0.1 mM-cysteine for 1 h at 15°C) (*a*) Field at low magnification. (*b*) Selected particles in which two monomers are connected. (*c*) Monomers with globular units at one or both ends. The bar represents 100 nm.



EXPLANATION OF PLATE 6

Electron micrographs (rotary shadowing) of extensively reduced intima collagen (20 mM-dithioerythritol for 24 h at 15° C) (*a*) Field at low magnification. (*b* Selected particles at higher magnification. (*c*) Type III pN-collagen shadowed under similar conditions shown for comparison. The slight difference in the diameter of the components in (*b*) and (*c*) is apparently due to a difference in the grain size. The bar represents 100 nm.

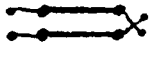
the two sides point in the same direction away from the axis of the particle. This feature is less clearly revealed by rotary shadowing, but a close inspection of Plate 1 confirms that the inner globular structures are somewhat displaced from the axis. Tetramers show two inner protuberances at both sides that point outwards in opposite directions, giving rise to a propeller-like appearance. Although the domains that form the 'propeller' are close to each other, a dark gap filled with stain is seen between them. This confirms the result obtained by rotary shadowing that there is not direct interaction between the inner globular domains of the two dimers in a tetramer. On the other hand the rod-like inner segments of the two dimers are closely associated in negatively stained tetramers. In this respect there is a difference between the results of the two electron-microscopic methods. Rotary shadowing produces images of tetramers in which the inner rod-like regions are clearly separated (Plates 1*c* and 1*d*), and only a few species that resemble the compact form observed by negative staining are observed (Plate 1*c*). Also, the outer rods with their small terminal globules are more closely aligned in negatively stained than in shadowed tetramers, but the scissors-like intersections can still be seen in many instances. More clearly than rotary shadowing, negative staining reveals a superhelicity of the inner rod-like regions of dimers and tetramers (Plate 2). Four to five intersections of the two structures can be counted per distance (75 nm) between the inner globular units.

The dimensions of dimers and tetramers are summarized in Table 1. In addition to the quantitative determination of dimensions, the fractions of the various types of dimers and tetramers were estimated by inspection of fields with a total of more than 200 particles. Results are summarized in Table 2. A similar electron-microscopic study to that described for intima collagen form A was performed for form B. The images of dimers and tetramers of form B were indistinguishable from those obtained for form A. Also, all dimensions agreed within limits of error. The only difference was in the distribution of species, which information is therefore included in Table 2.

Table 1. *Dimensions of intima collagen and its fragments as determined by the rotary-shadowing technique*
Intima collagen forms A and B, their Col fragments and materials obtained by extensive reduction and alkylation under non-denaturing conditions (NRA) were prepared as described by Odermatt *et al.* (1983). *l* is the distance between the centres of the inner globular units for collagen forms A and B and their Col fragments and the length of strands for collagen A Col fragment (NRA). *a* is the distance between the centres of the inner and the nearest outer globular unit. *L* is the length of the strands for collagen forms A and B (NRA) and the sum of *l* and *a* for collagen forms A and B. The error limits are standard deviations.

Component	No. of particles analysed	*l* (nm)	*a* (nm)	*L* (nm)
Intima collagen form A	120	75.4 ± 4.8	29.2 ± 3.3	104.6 ± 8
Intima collagen form B	50	74.4 ± 7.1	28.7 ± 4	103.1 ± 11
Intima collagen A Col fragment	110	74.2 ± 3.5	—	—
Intima collagen B Col fragment	30	78.5 ± 4.5	—	—
Intima collagen form A (NRA)	140	—	—	105 ± 9
Intima collagen form B (NRA)	22	—	—	102 ± 12
Intima collagen A Col fragment (NRA)	30	78.8 ± 5.0	—	—

Table 2. *Distribution of particles found in electron micrographs (rotary shadowing) of intima collagen forms A and B*
A small fraction of higher aggregates was ignored. Only particles that could be clearly classified were counted. These were about 70% of all particles in a field. The total number of particles was 218 for collagen form A and 205 for collagen form B.

Schematic drawing	Identification	Fraction: Intima collagen form A	Fraction: Intima collagen form B
	Dimer	0.42	0.41
	Dimer, inner part unravelled	0.04	0.26
	Dimers, with only one outer rod	—	0.12
	Tetramer, both outer rods connected	0.39	0.11
	Tetramer, connected at one side only	0.14	0.10

Fibrous structures

Both rotary shadowing and negative staining revealed structures that consist of several tetramers. In many cases these structures are probably caused by random association, a phenomenon often observed in electron microscopy. In particular, aggregates without common structural features appeared at the edge of a grid at interfaces of droplets originating from spraying the solution for rotary shadowing. There was, however, a linear 'fibrous' structure with a clear periodicity, which was often seen even in the central regions of fields and at low particle densities (Plate 3). Most probably these structures reflect a mode of association that was already present in solution. Two or more tetramers are linked by their outer rod-like segments. The spacing between the inner globular units (propellers) of adjacent tetramers in negatively stained material is 42nm, thus clearly exceeding the length of the outer rods (30nm). Small globular units are visible in the connecting region at a distance of 10–15nm from the nearest 'propeller' units. This suggests an anti-parallel incomplete overlap of the outer rods of the two interacting tetramers. Rotary shadowing reveals a qualitatively similar arrangement, but the corresponding spacing is 30nm instead of 42nm, suggesting a complete anti-parallel overlap of the outer segments.

Fragment Col obtained with bacterial collagenase

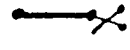
Electron micrographs of this fragment prove that collagenase removed the outer rod-like segments of intima collagen. Most of the particles (75%) of fragment Col of form A that were subjected to rotary shadowing (Plate 4) have the appearance of dumb-bells (Plate 4*b*). The particles resemble in length (75nm) and other details the inner rod-like regions of intima collagen (see Table 1). The inner globular domains are at least partially maintained. In a significant fraction (see Table 3) only one of the outer rods with the outer globes was removed (Plate 4*c*), and 3% of the particles retained both outer rod segments and are identical with dimers of intima collagen. A few particles contain a scissors-like structure attached to a dumb-bell. These are interpreted as incompletely digested tetramers. A significant fraction of the fragments exhibited complete or partial strand separation (Plate 4*d*). This confirms the observation described above for intact intima collagen. A superhelicity of the double-stranded rod-like regions is suggested on replicas of well-resolved particles at higher magnification (Plate 4*e*). This finding is confirmed by negative staining (Plate 2*c*). As observed already for the inner rod-like region of intima collagen, there are at least two full superhelical turns. The number of turns may be higher because partial or complete untwisting is often observed (Plates 2 and 4). It should be noted that even when the strands are completely unravelled they remain connected at the ends.

Fragment Col prepared from intima collagen form B was electron-microscopically indistinguishable from that of form A. As in the case of intact intima collagen, the distribution of particles varies somewhat, but these differences may also reflect small differences in the collagenase treatment and purification. It is remarkable that in electron micrographs of fragment Col (form A or B) no particles are detectable that are larger than those described. The removal of the outer segments apparently results in the complete dissociation of aggregates.

Fragments produced by reduction

Very mild reduction (0.1mM or 1mM-cysteine for 1h at 15°C) under native conditions produces strands with a globular unit at one end (Plate 5) and a length of 105nm (Table 1). A fraction of the particles (about 10%) showed in addition a smaller globular unit at the other end. The apparent diameter of the threads is comparable with that of pN-collagen III molecules when subjected to rotary shadowing under the same conditions (Plate 6*c*). It may therefore be concluded that the thread-like portion of the reduced and alkylated fragments consist of a single triple helix. Most of the particles (60%) are connected via the larger globular domain to the triple helix of a second strand (Plate 5). The site of attachment to the triple helix in these dimers is

Table 3. *Distribution of particles seen in fields of rotary-shadowed Col fragment (form A)*

Schematic drawing	Identification	Fraction
	Dumb-bell, e.g. inner segment of intima collagen	0.62
	Two triple helices of inner rod-like segment partially or completely unravelled	0.13
	Only one outer rod-like segment removed	0.19
	Intact dimers	0.03
	Partially degraded tetramers	0.02

30nm apart from the end that carries the smaller globular unit or at which no morphological detail is visible.

When the time of reduction was increased or when a stronger reducing agent was used, the fraction of dimers decreased, and at the same time strands of 105nm length but without globular units became apparent. For example, treatment with 2mM-dithioerythritol at 15°C for 30min produced a distribution of strands in which only about half showed a globular unit at one end. Only very few particles exhibited two globular units, and only a small fraction (about 5%) of dimers of the type shown in Plates 5(*b*) and 5(*c*) were seen. After extensive reduction (20mM-dithioerythritol for 24h at 15°C) a rather homogeneous population of strands of length 105nm lacking clear morphological details at their ends was observed (Plate 6).

By extensive reduction of fragment Col particles were obtained that exhibited the length of the original fragment (Table 1) but that according to its apparent diameter consisted of a single triple helix. No clear globular units were detectable by the rotary-shadowing technique. Electron micrographs (not shown) of the reduced fragment resembled those of extensively reduced intima collagen (Plate 6), except that the strands were about 30% shorter.

Flexibility of intima collagen fragments

The electron micrographs of the triple-helical regions of the fragments show considerable curvature (see Plates 1–6). A quantitative determination of the flexibility was achieved by a method (H. Hofmann, T. Voss, H. Wiedemann, K. Kühn & J. Engel, unpublished work) that is an extension of the methods of Takebayashi *et al.* (1977) and Frontali *et al.* (1979). Essentially the mean-square curvature is measured at any point of the molecules from measurements of the local curvature of a large number of molecules. The flexibility was found to be rather uniform along the entire triple-helical regions in reduced intima collagen and fragment Col. The finding of uniform flexibility justified an evaluation of the persistence length a from the mean-square end-to-end distances and the contour length of the molecules (Takebayashi *et al.*, 1977; Engel *et al.*, 1981). The values found were $a = 82$nm for reduced intima collagen and reduced fragment Col, but non-reduced fragment Col exhibited a value of 122nm. A persistence length of about 60nm is characteristic for the regions of uniform flexibility in collagens I and III (H. Hofmann, T. Voss, H. Wiedemann, K. Kühn & J. Engel, unpublished work). The 2-fold larger value for fragment Col is consistent with two triple helices aligned in parallel. According to simple mechanical considerations, such a structure should exhibit about double the stiffness as a single triple helix.

Molecular masses and sedimentation coefficients

The M_r value of reduced intima collagen that was produced by mild conditions (10mM-cysteine for 1h at 15°C) could be established with high precision. Addition of 1M-urea to the ammonium bicarbonate buffer prevented aggregation, and M_r 170000 ± 10000 was found for the native structure. The latter was verified by c.d. spectra and 'melting' profiles in the presence and in the absence of urea (Odermatt *et al.*, 1983), which indicated that 1M-urea does not cause unfolding of the protein at 20°C. The material sedimented as a homogeneous peak with $s^0_{20,w}$ 3.5 S. The minimum M_r of intima collagen after extensive reduction (20mM-dithioerythritol, for 24h at 15°C) was 160000 ± 30000. However, this preparation exhibited considerable aggregation even in 1M-urea.

In accordance with the electron-microscopically observed heterogeneity, equilibrium plots ($\ln A$ versus x^2) of intima collagen forms A and B were strongly curved, with apparent weight-average M_r values ranging from 300000 to 450000 at the meniscus to 600000 to 10^6 near the bottom of the cell, depending on rotor speed and preparation. The only meaningful information that can be deduced from the data is the minimum M_r, which was found to be 350000 ± 50000 near the meniscus at high rotor speeds. This value may be correlated with the particle weight of the dimer. For fragment Col a minimum M_r of 240000 ± 40000 was found. Within the limits of error values obtained for forms A and B of intima collagen were identical.

In sedimentation-velocity runs of intima collagen form A two boundaries of about equal size sedimenting with about $s^0_{20,w}$ 5S and 9.5S were detected. A significant fraction of the material (about 30%) sedimented with higher rates, but no distinct sedimentation profiles were apparent. We attribute the 5S profile to dimers, the 9.5S profile to tetrameric species and the faster-sedimenting material to higher aggregates. For intima collagen form B the same profiles were seen, but the slower profile (5S) was larger than the faster one (9.5S). This is in accordance with the larger fraction of dimers in electron micrographs of form B as compared with form A (Table 2). Fragment Col obtained from both forms A and B sedimented with $s^0_{20,w}$ 4.8S. All sedimentation coefficients were obtained by extrapolation to zero concentration. Because of the high concentration-dependence, error limits are unusually high (±10%).

The measured sedimentation coefficients and molecular masses were compared with values calculated from the electron-microscopically determined dimensions (Table 4). The molecular masses of the collagen-like parts of the molecules could be determined from the lengths of these regions and the molar mass-to-length ratio $M/L = 1000\,\text{nm}^{-1}$ of

Table 4. *Comparison of molecular masses and sedimentation coefficients with values calculated from electron-microscopically observed dimensions*

Component	Collagen part $10^{-3} \times M_r$	Inner globular domain r (nm)	Inner globular domain $10^{-3} \times M_r$	Outer globular domain r (nm)	Outer globular domain $10^{-3} \times M_r$	$10^{-3} \times M_{r(total)}$ Calculated	$10^{-3} \times M_{r(total)}$ Observed	$s^0_{20,w}$ (S) Calculated	$s^0_{20,w}$ (S) Observed
Intima collagen Col fragment	146*	1.81 2.07 2.28 2.46	20 30 40 50	—	—	186 206 226 246	240 ± 40	4.4 4.8 5.2 5.5	4.8 ± 0.5
Intima collagen dimer	200†	2.28	40	1.65 2.07	15 30	310 340	350 ± 50	5.1 5.45	5.0 ± 0.5
Intima collagen tetramer	400	2.86	2 × 40	2.07 2.61	2 × 15 2 × 30	620 680	—	9.0 9.5	9.5 ± 0.5
Intima collagen monomer	103	2.28	40	1.65 2.07	15 30	148 173	170 ± 10	3.1 3.4	3.5 ± 0.2
Collagen I	295	—	—	—	—	295	295	2.8	2.9 ± 0.2‡
Procollagen I	295	—	—	2.46 3.10	50§ 100‖	545	545	3.9	4.2¶
pN-collagen III	295	—	—	2.46	50§	345	345	3.15	3.2 ± 0.2**

* Calculated from length between inner globular units (73 nm) and $M/L = 2000\,\text{nm}^{-1}$.
† Inner part with two triple helices (M_r 146000) plus two outer single-helical segments ($M_r = S \times 27 \times 1000$).
‡ Engel & Beier (1963).
§ *N*-Terminal propeptides.
‖ *C*-Terminal propeptides.
¶ Fiedler-Nagy *et al.* (1981).
** H. P. Bächinger & J. Engel (unpublished work).

a collagen triple helix. The lengths of the collagenous regions were taken from Table 1, with application of small corrections for the diameters of the globular units. The size of the globular units can be determined from electron micrographs with considerably less precision than the lengths of the collagen rods. On rotary-shadowed replicas all diameters appear too large, owing to the decorating effect. A rough estimate is, however, possible by comparison with known structures viewed under identical conditions. The inner globular units of the dimer have about the same apparent diameter as the *N*-terminal propeptide (M_r about 50000) of pN-collagen III (Plate 6). The outer globular units are apparently smaller. By negative staining the inner globular units appear as prolate spheroids with long and short half-axes of 5 nm and 3 nm respectively. These dimensions would correspond to an M_r value of 190000. This is difficult to reconcile, however, with the observed total molecular masses and sedimentation coefficients.

More reliable estimates can possibly be derived from hydrodynamic data. The sedimentation coefficient of rod-like particles is quite sensitive to the size of the globular units attached to the ends. It is therefore possible to determine permissible ranges for the molecular masses of the globular domains by calculating theoretical sedimentation coefficients. The method of Bloomfield *et al.* (1967*a*,*b*) was applied. For a construction of hydrodynamic equivalents the inner and outer globular domains were represented by spheres of radius:

$$r = \sqrt[3]{\frac{3 \cdot v \cdot M_r}{4 \cdot \pi \cdot N_A}}$$

where N_A is Avogardro's number, v (= 0.73 ml/g) is the partial specific volume of an average protein and M_r is the molecular mass of the domain. M_r was varied in a reasonable range (see Table 4). For the sake of simplicity the two adjacent globular units of the composite dimers in the hydrodynamic model of the tetramer were combined in a single sphere with a radius calculated from the sum of the molecular masses of the two domains. This model closely represents the shape of tetramers seen by negative staining. The collagen-like segments were represented by rows of spheres with radii 0.75 nm for single triple helices and 1.06 nm for two parallel triple helices. With this choice of radii the M/L ratios of the rows of spheres are $1000\,\text{nm}^{-1}$ and $2000\,\text{nm}^{-2}$ respectively, assuming a partial specific volume of 0.71 ml/g for collagen.

Molecular masses of the inner globular units of 30000 to 40000 are consistent with the measured

sedimentation coefficient of fragment Col, and values lower than 20000 and higher than 50000 can be ruled out (Table 4). With a value of 40000 a molecular mass of 15000 follows for the outer globular domain from a comparison of calculated and observed sedimentation coefficients of the dimer. Values for reduced intima collagen in which the globular domains were preserved by mild reduction are in better agreement with a somewhat larger outer domain (M_r 30000 instead of 15000) (Table 4). It was also verified by the Bloomfield models that the sum of the molecular masses of the inner and the outer units in the dimer must be in the range of 40000–70000 in order to be consistent with a sedimentation coefficient of 5 ± 0.5 S. The sedimentation coefficient that was calculated for the tetramer as a lateral aggregate of two dimers agrees well with the observed value (Table 4).

As a test for the validity of the hydrodynamic calculations, the sedimentation coefficients of collagen, procollagen and pN-collagen were calculated by the same method. Good agreement with available experimental values is observed (Table 4).

Discussion

A rather detailed model of the structural organization of intima collagen can be derived by combining the information obtained for the different fragments. The monomeric unit, which is obtained by reduction and alkylation under non-denaturing conditions, consists of a triple helix about 105 nm in length with globular domains of different sizes (M_r about 30000 and 40000) at its ends (Fig. 1). This triple helix contains no kinks or obvious sites of increased flexibility, and its persistence length is comparable with those of other collagen triple helices.

The next higher form of organization is a dimer formed by lateral association of two monomers arranged in anti-parallel fashion with a stagger of 30 nm. Dimers are probably stabilized by weak non-covalent interactions between the two triple helices within the overlapping region. These may be responsible for the formation of a two-stranded superhelix (Fig. 1).

Decisive for the interconnection of the monomers

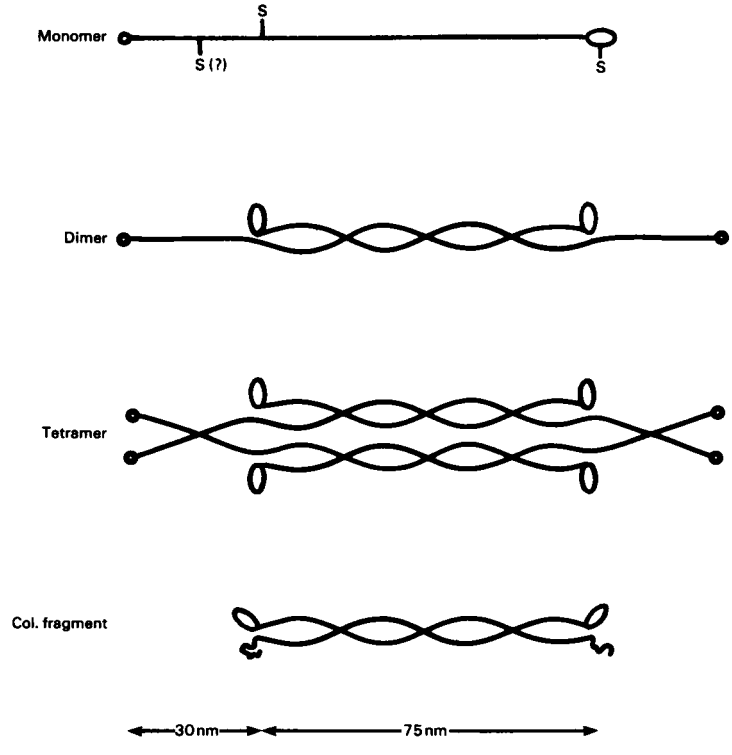


Fig. 1. *Schematic drawing of the structures of monomers, dimers and tetramers of intima collagen and of Col fragment* S indicates the tentative localization of half-cystine residues that participate in disulphide bridges between monomers in dimers and tetramers.

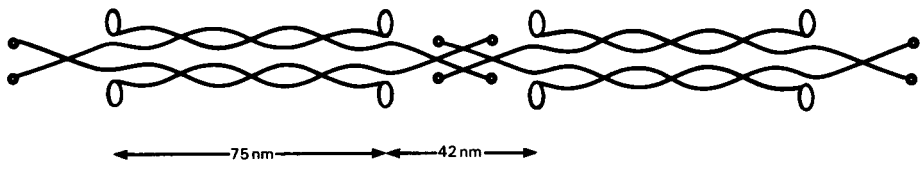


Fig. 2. *Model of a filamentous form of intima collagen*

are disulphide bridges, which are apparently located in the regions of the inner globular domains. Strand separation was often observed within the central region, but never near the inner globular units. This localization of the disulphide bridges has been confirmed for fragment Col, which constitutes the central portion of the dimers. Again strand separation was not observed at the ends, whereas various kinds of loops were seen within the central region. Chemical analysis showed that complete dissociation of the dimer is achieved after reduction of only two disulphide bridges (Odermatt *et al.*, 1983). This suggests the presence of a single disulphide bridge between each of the inner globules and the adjacent triple helix in the dimer. A striking property of these disulphide bridges is their unusual sensitivity towards low concentrations of weak reducing agents such as 0.1–10 mM-cysteine. Partial reduction under very mild reducing conditions led to dimers in which monomeric units are linked at one site only. These products conclusively demonstrated the staggered arrangement of monomers into a dimer. The large number of cysteine residues that are reduced under more drastic conditions only (Odermatt *et al.*, 1983) apparently form intramolecular bridges, and some of them may serve to stabilize the globular domains. This would explain why after extensive reduction the globular units are no longer seen on electron micrographs. The peptide extensions may be partially removed by extensive reduction or rendered invisible by unfolding.

Tetramers are formed from dimers, probably by disulphide bridging within the scissors-like regions (Fig. 1). Since preparations of intima collagen contain a substantial fraction of tetramers with one open end and of free dimers, this bond may not have formed all the time. Non-covalent interactions between the central portions of the composite dimers may also contribute to the stabilization of the tetramers. Electron micrographs, particularly of rotary-shadowed specimens, frequently show the contacts in this region to be disrupted. The tetramer appears to be the building block of fibrillar structures (Fig. 2) formed by interaction between the outer triple-helical portions and the outer globular domains. The exact nature of the end-to-end linkage in the fibrous forms is not clear.

By comparing intima collagen with other types of collagens, it is apparent that the structure of the monomer in general resembles the structure of an interstitial procollagen in which the central triple helix separates two globular domains known as the *N*-terminal and the *C*-terminal propeptides (Fessler & Fessler, 1978). Recent studies have shown that partially processed procollagens or procollagen-like molecules are important matrix components. These include pN-collagens I and III in thin collagenous fibrils (Fleischmajer *et al.*, 1981), the globular domains of collagen IV promoting their interaction within basement membranes (Timpl *et al.*, 1981) and perhaps another globular domain unique to the matrix form of collagen V (Kumamoto & Fessler, 1980, 1981; Foidart *et al.*, 1981). Thus intima collagen is another example of a collagenous protein requiring globular domains for maintaining a distinct matrix assembly.

The particular shape and association of intima collagen indicates that it is derived from a unique protein not related to the known collagen types I–V. We propose therefore to refer to it as a new collagen type VI. The potential for end-to-end aggregation and the bulky nature of the globular domains, which should interfere with lateral aggregation of the tetramers, also suggests that intima collagen originates from a microfibrillar component. Microfibrils of 10–20 nm diameter are particularly found at the interface between elastic and collagenous fibrils (Desgranges *et al.*, 1976; Serafini-Fracassini *et al.*, 1977; Jones *et al.*, 1980), and are thought to contain a collagenous component (Sear *et al.*, 1981). This component is composed of chains with M_r 150000 that are similar in composition to intima collagen (Gibson & Cleary, 1982). A relationship may also exist to EC collagen, produced by cultured endothelial cells (Sage *et al.*, 1980), which is degraded by pepsin to chains of M_r 50000. The particular electron-microscopical approach developed in our present study should be helpful for clarifying the possible relationship between intima collagen and other potential microfibrillar collagen components.

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