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AXIAL MASS DISTRIBUTIONS OF COLLAGEN FIBRILS GROWN IN VITRO: RESULTS FOR THE END REGIONS OF EARLY FIBRILS

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Summary The growth of native type fibrils from acetic-acid-soluble collagen has been investigated by quantitative dark-field electron microscopy. Axial mass distributions of early D-periodic fibrils were measured and expressed as plots of n_a against axial position (n_a is the number of molecules in a fibril cross section). Measurements were made on entire fibrils in the length range 15 D to 250 D. At the end regions of fibrils these plots were linear, with n_a increasing by 4 - 5 molecules per D-period at the N-terminal end and 8 - 10 at the C-terminal. These results were not dependent on fibril length and were observed over a range of precipitating conditions.

Introduction The mechanism of collagen fibril assembly is unknown. Although it has been treated as a simple nucleation/growth phenomenon (1), recent electron microscope studies (2-4) and solution measurements (5-7) indicate that assembly *in vitro* involves multiple steps and that the species accreting on to the growing fibril is not monomeric. Electron microscope observations in this laboratory (8,9) are also inconsistent with the simple 'nucleation/growth at constant shape' model (1).

The present paper describes a size/shape study of fibril growth at a stage at which fibrils are long enough to display D-periodic stain banding but are not so long that both ends cannot be seen (so-called 'early' fibrils, observed when < 1% of collagen has precipitated). Up to now electron

The following abbreviations are used:
D, D-period: the periodic repeat in collagen fibrils, caused by regular axial staggering of molecules,
n_a: the number of collagen molecules in a fibril cross section, averaged over one or two D-periods,
X: the axial distance along a fibril, expressed in D-periods,
AMD: axial mass distribution, i.e. the mass per unit axial length of a fibril as a function of X,
L: the over-all length of a fibril, expressed in D-periods.
Other abbreviations in Materials and Methods are defined as they are used.

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microscopy of fibrils grown *in vitro* has provided only approximate size/shape data. Distortion on drying on a support film (so that transverse shape is unknown) makes width measurements unreliable. No method used hitherto has allowed simultaneous measurement of transverse size and axial position with accuracy.

A far more accurate measure of these parameters can be obtained by quantitative dark-field electron microscopy. It has been used here to study the growth characteristics at the ends of 'early' fibrils.

Materials and Methods Acetic-acid-soluble calf skin collagen was prepared as described (10) and stored deep-frozen in 0.01M acetic acid. The amino acid composition was characteristic of this type of acid-soluble collagen (11). The collagen concentration was 950 ± 20µg/ml. This was derived from a determination of the hydroxyproline concentration (12) assuming 13.6 % w/w in collagen (13).

'Standard' conditions for fibril formation were an I 0.2 KH_2PO_4/Na_2HPO_4 buffer, pH 7.4, at 34°C and a collagen concentration of 95 µg/ml. Precipitation was initiated by mixing the collagen solution in 0.01M acetic acid with buffer solution. Both solutions were at twice the required final concentrations and were pre-warmed to the precipitation temperature.

Sampling the collagen solution about one minute after initiation of precipitation gave convenient numbers of 'early' fibrils. A drop of solution was deposited on a carbon-filmed grid and flushed immediately with a few drops of buffer having the composition and temperature of the precipitating buffer solution. Failure to adopt such a procedure led to the presence of large quantities of finely-filamentous material on the carbon film. The buffer was subsequently washed away with doubly-distilled water.

Uranyl acetate (pH 4.2) alone or sodium phosphotungstate (pH 3.5) followed by uranyl acetate were used as stains to establish fibril polarity. Otherwise specimens were unstained.

Procedures for mass determination by quantitative dark-field electron microscopy have been described by Brakenhoff *et al* (14). In the present study an AEI EM6B instrument was used; the accelerating voltage was 80kV. An off-axis 20 µm objective aperture produced dark-field imaging. Linearity between dark-field image intensity and specimen mass thickness was established over the range of specimen thickness used. The two specimen types (collagen and the TMV used as a standard) were effectively amorphous as indicated by electron diffraction under the same illuminating conditions.

A scanning microdensitometer with digital output (Joyce Loebl Autodensidater 3CS) was used for densitometry of fibril micrographs. A series of scans were made transverse to the fibril axis. The length of the slit was set to 2 D-periods and the width was small compared with the width of the fibril image.

Tobacco mosaic virus was used as an internal standard of mass per unit length. The dry mass per unit length of TMV (m_{tmv}) and of a single collagen

molecule (m_{cm}) are known to within about 4 %; the values used here are $m_{tmv} = 1.3 \times 10^5$ Daltons nm^{-1} (15) and $m_{cm} = 1.0 \times 10^3$ Daltons nm^{-1} (16). The experimentally-observed quantity (from electron microscope measurements) is $r_{cf/tmv}$, the ratio of the measured dry mass per unit length of the collagen fibril to that of the TMV rod (both after electron exposure). Allowing for shrinkage and mass loss, the number, n_a, of molecules in a fibril cross section is then:

$$n_a = r_{cf/tmv} \cdot (m_{tmv}/m_{cm}) \cdot f_s \cdot f_m.$$

The axial shrinkage factor f_s is given by D'/D, where D' is the observed periodic length of the dried fibril specimen and D that of the fibril in the native state. An average value of D' was measured from the dark-field micrograph of the unstained fibril; a value of D = 66.8 nm was used (17). Magnification was determined in each case using a diffraction grating replica.

The relative mass loss factor f_m is the ratio of the fractional mass of the TMV standard remaining after electron exposure to the corresponding fractional mass residue of the fibril specimen following similar exposure. Individual fractional mass losses of biological specimens may be as high as 0.4 at 80kV (18,19) but use of TMV as an internal standard may be expected to minimise errors due to mass loss. That f_m is near unity was confirmed experimentally using quantitative bright-field electron microscopy (20). (At a given electron dosage of the specimen, bright-field here, with a 20 µm objective aperture, gives greater accuracy in relative mass measurement than dark field.) Mass measurements over a 100-fold range of electron dosage indicated fractional mass losses between 0.15 and 0.25 for a similar electron exposure to that used in the dark-field mass determination measurements (about 1.6×10^{-4} A cm^{-2} for 6 sec). No significant difference in the behaviour of collagen and TMV was observed. Our results show that taking f_m as unity gives an error in n_a no greater than about 10%.

Relative mass per unit length measurements on positively stained fibrils were made by quantitative bright-field electron microscopy using 30 keV electrons and a 20 µm objective aperture in the AEI instrument. These measurements were used to compare the polarity of the axial mass distribution with the molecular polarity of the fibril.

Results and Discussion Typical axial mass distributions (AMDs) in the form of plots of n_a (the number of molecules in the fibril cross-sectional area averaged over 2 D-periods) against X (the distance along the fibril axis in D-periods) are shown in Fig. 1. Entire AMDs were measured for fibrils in the length range 15 D to 250 D; the upper limit was that for fibrils in which both ends could be observed.

A distinctive feature of the AMDs of these 'early' fibrils was a near-linear dependence of n_a on X in the terminal regions. For each fibril, however, the AMD was asymmetric with different slopes at the two ends. These two slopes were the same for almost all fibrils, irrespective of length. The

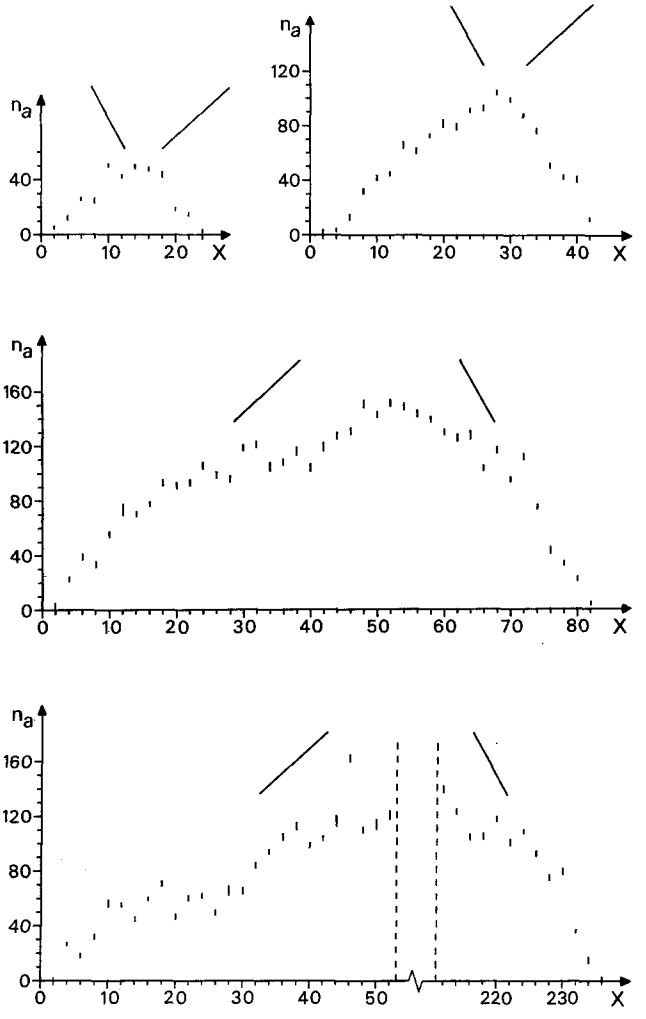


Fig. 1. Typical axial mass distributions (AMDs) for early fibrils determined by quantitative dark-field electron microscopy. n_a, the number of molecules in a fibril cross section, averaged over 2 D-periods, is plotted against X, the distance along the fibril axis in D-periods. The error in each n_a value has been estimated from the background noise level in the corresponding densitometric scan. The lines above have slopes of 4.5 and 9 molecules per D-period.

smaller slope corresponded to an increase in n_a of 4 - 5 molecules per D-period, the greater slope to 8 - 10 molecules per D-period; linearity persisted over about 20 D and 10 D at the two types of end respectively. Slope data obtained by least squares fits over these linear regions of the AMDs are summarised in Fig. 2.

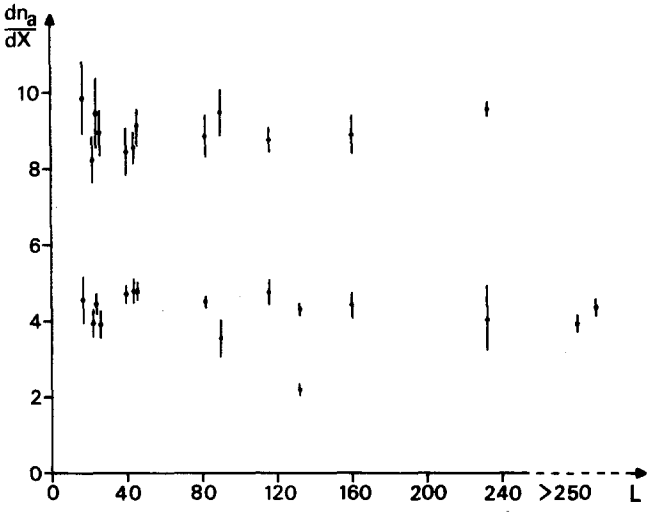
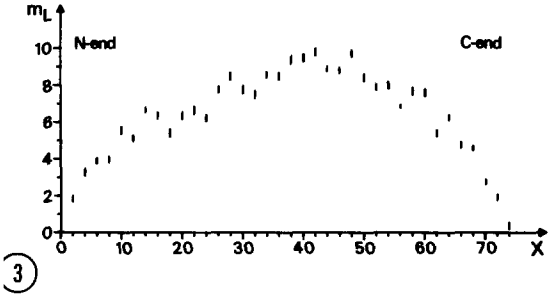


Fig. 2. Summarised terminal data from 15 fibrils. Terminal slopes (dn_a/dX molecules per D-period) from AMDs are plotted against L, the fibril length in D-periods. Slopes have been derived from linear least squares fits. The two ends of each fibril give rise to two points for each value of L. The two points for L > 250 D are from two fibrils for each of which only one end could be found.



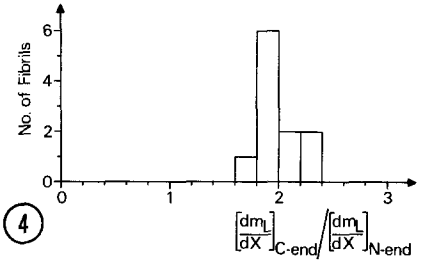


Fig. 3. Relative AMD of an early fibril stained with phosphotungstate/uranyl, determined by quantitative bright-field electron microscopy. m_L, the relative mass per unit length (with contributions from collagen and stain) expressed in arbitrary units is plotted against X, the distance along the fibril axis in D-periods. The molecular polarity of the fibril has been established from the stain pattern.

Fig. 4. Terminal data from 11 positively stained fibrils (in the length range 50 - 90 D). The histogram shows the frequency of occurrence of the measured ratio (slope of relative AMD at C end/slope of relative AMD at N end) for individual fibrils.

Quantitative electron microscopy of positively stained fibrils yielded relative AMDs demonstrating a similar slope asymmetry. Fig. 3 shows this asymmetry in a fibril stained with PTA and UA. Using the staining pattern to establish the molecular polarity of fibrils, a consistent correspondence

was found between AMD asymmetry and fibril polarity (Fig. 4). The 'blunt' end (the one of greater slope) of each relative AMD corresponded to the C-terminus of the fibril.

Our evidence indicates that the number of molecules in a cross-section of an early fibril increases with each D-period by 4 - 5 at the N-terminal and 8 - 10 at the C-terminal. These increases are maintained over distances of about 20 D-periods and 10 D-periods respectively. This growth behaviour is substantially insensitive to solution conditions in the following ranges: collagen concentration 12 - 190 µg/ml; ionic strength I 0.1 - I 0.5 (adjusted with NaCl in I 0.01 phosphate buffer); temperature 22.5 - 34 °C. (At the lowest limits of collagen concentration and of temperature however, fibrils of length > 90D did show increased slopes in the AMDs of the end regions; for fibrils formed at 12 µg/ml slopes up to 25 and 12 molecules/D-period were found; for fibrils formed at 22.5 °C slopes ranged up to 20 and 10 molecules/D-period.) These precipitating conditions all gave rise to native-type D-periodic fibrils. Fibrils precipitated in solutions for which I > 0.5 contained a D-periodic-symmetric component (21).

Asymmetry in the N- and C-terminal growth behaviour of collagen fibrils has also been demonstrated by Haworth and Chapman (22) for fibrils of length 30 to 180 µm (i.e. 450 - 2,700 D-periods); the average axial growth rate of the N-terminus was found to be greater than that of the C-terminus by a factor varying from 1.2 to 1.7.

Finally, our results indicate a diameter-retarding process at this early stage of fibril growth. They show that the rate of accretion on to unit area of the fibril surface (relative to the axial growth rate) decreases with increasing cross-sectional size. For a fibril of circular cross section growing axially at a uniform rate, a linear dependence of n_a on X in the end regions implies that the radial growth rate ($\partial R/\partial t$) is inversely proportional to the radius (R) in these regions. Further data on growth behaviour will be given in a later publication.

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