Ultrathin supporting films in electron microscopy of unstained biological macromolecules

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Summary. — A simple technique is described, in which the increase in thickness of a carbon film is controlled. Films up to 10–15 Å in thickness, which are both continuous and strong enough for use as supporting films in electron microscopy, can be easily obtained by this routine method.

Images are shown of unstained ribosomal subunits and tropocollagen molecules on 15 Å carbon films.

INTRODUCTION

Contrast in electron microscopy images of biological molecules or small molecular aggregates is extremely low, due to the unavoidable presence of the supporting film, which contributes to the three effects which appear to be superimposed in the formation of these images: elastic scattering, phase contrast, inelastic scattering.

Carbon films 100–200 Å thick are generally used as supporting films; but these are too thick if biological molecules with dimensions of only 10–20 Å are observed. Moreover, they are usually obtained by arc discharge between two carbon electrodes; by this technique the strata are not homogeneously produced, due not only to their zonal structure (KAKINOKI et al., 1960), but also to the frequent deposition on them of graphite microcrystals emitted from the source. These are easily visible under the electron microscope, if cross sections of carbon films (embedded in araldite and cut in the ultramicrotome) are observed.

In this paper a simple routine method is described for the preparation of ultrathin films (up to 10–20 Å in thickness), which are continuous and strong enough for use as supporting films in high resolution electron microscopy of low atomic weight material.

Examples are given of images of unstained biological macromolecules laid down on films about 15 Å thick.

MATERIALS AND METHODS

Shadowing plant.

A Speedivac shadowing apparatus (Edwards High Vacuum Ltd., 12 E6/1707) was used, equipped with the electron gun described by Chopra and Randlett (1966) source and a liquid air trap between the chamber and the oil diffusion pump. As showed in Fig. 1, the work chamber of the apparatus was lengthened by means of a metal cylinder with a water cooling system, composed of a copper tube sealed around the cylindrical wall. A glass window was sealed into this wall and two rods were inserted to enable the screens to be moved into position. After which, the total length of the bell jar measured 60 cm, and the work chamber could be pumped down to $10^{-3}$ torr in 5 min. The glass or mica surface used to support the carbon

![Fig. 1. Scheme of the work chamber: the lengthening metal cylinder with the water cooling system and the window, the electron gun, the screens and the film supporting surface are outlined.](image-url)
film was 15.6 cm far from the emitting point of the carbon electrode. The temperature of the supporting surface, measured with a copper vs constantan thermocouple, never rose above 125 °C, even after several hours of evaporation.

The supporting surface was rotated at approximately 7 RPM, normally to the axis of the system, in order to ensure more uniform deposited layers.

The geometry of the system and the working conditions of the electron gun were rigorously standardized; an external electrometer (3440 Hewlett-Packard digital electrometer) was inserted to increase the accuracy of readout, so that the current in the gun could be maintained between 18.5 and 19.5 mA with 4 kV as a high voltage. Under these conditions no sparking from the source occurs.

The apparatus was accurately outgassed before evaporation, the electron gun being excited. The carbon deposition started when the vacuum reached 2.10⁻³ torr: this pressure lasted throughout the entire period of deposition.

**Carbon electrode.**

Spectroscopically pure carbon rods were obtained from Ringsdorff-Werke GMBH (Godesberg), as graphitic carbon rods (RW1). Even if amorphous carbon would be preferable to the graphitic type on account of its higher resistivity (60Ω mm²/m, instead of 8 Ω mm²/m), its property to absorb large quantities of gas and vapours makes it unsuitable for use in the vacuum plant. The rods were 6.15 mm in diameter; one of their tips was sharpened by lathe to a 60° point, the end being reduced to 1.2 mm diameter. The total length of the carbon electrode measured 29 mm.

**Supporting surface.**

The carbon film for use in the interferential microscope was formed on glass slides, whilst that for use under the electron microscope was formed on freshly cleaved mica. The method (introduced in our Laboratory by A. K. Kleinschmidt) of covering a glass slide with a thin collodion film and using it as a supporting surface for the carbon film is unsuitable, as the collodion backing the film is never completely destroyed in the oven, even after several hours at 200 °C.

To check this point a specimen grid was covered by a carbon film and a collodion layer was laid down on a portion of the carbon film. The observations before and after the oven treatment showed that the borderline of the collodion film was clearly visible in both cases. On the contrary, the collodion film alone, unbacked by the carbon film, was easily destroyed. The chemical treatment with a mixture of alcohol and ether 50:50 was able to destroy the collodion film backed by the carbon in 10 sec, but only strong carbon films could endure the strain due to this procedure.

Carbon films no less than 100 Å in thickness can easily be detached from the mica surface, on account of their mechanical resistance, which is comparatively high: below this limit, care must be taken in handling them, and below 50 Å it is very difficult to detect the film when it floats off the mica. It is easier to detach and to see if a heavier carbon evaporation is made on an edge (Williams & Glaeser, 1972).

Supporting grids.

All the films obtained, up to 10–15 Å thick, could be picked up by merely touching them, from the air side, with a 400 mesh copper grid.

Thickness measurements.

A Leitz transmission interferential microscope was used in monochromatic light (\(\lambda = 5460 \, \text{Å}\)), and the half-shade method was applied (Smith, 1954; Pluta, 1971; Scott, 1971). Some controls were made with a Wild reflection interferential microscope.

To avoid scattered light from the glass slide, the 400-mesh grid covered by the film under measurement, was supported by a perforated metal plate. The measuring was made in a uniform field: a portion of a square of the grid which was partially covered by the film, and in which a bar appeared, was centered in the microscope. The phase compensator was set first of all so that the film and the bar of the grid showed exactly the same brightness, and then, the second time, with the background (i.e. the zone uncovered by the film) showing the same brightness as the bar. The two readings give the optical path in the film, if calibration of the compensator system has been made.

To evaluate the thickness of the film, the refractive index of the carbon film must be known. This value can be easily obtained if, in addition to the transmission interferential microscope, a reflection interferential microscope is available: in our case thickness measurements were made both with the Leitz and the Wild apparatus on a film about 500 Å thick, by the fringe-field method.

The optical path in the carbon film \(\delta_c\) was first measured under the transmission interferential microscope, and then the optical path in a thickness of air equal to that of the carbon film was measured under the reflection differential microscope. To enhance the reflectivity of the stratus a silver film was evaporated both on the carbon film and on the glass surface supporting it. The difference in the optical path of the light beam (\(\lambda = 5460 \, \text{Å}\)) reflected on the carbon film and that reflected on the supporting surface gives twice the optical path \(\delta_s\) in the air, corresponding to the thickness of the film. It is easily shown that the refractive index is given by \(n = \delta_c/\delta_s + 1\).
By this method, measurement for the refractive index of carbon film gives $n = 2.5$. Correspondingly the minimum thickness measured by the half-shade method on a carbon film is $22 \pm 15$ Å. It is not easy, however, to reach this limit, on account of the difficulty in judging with sufficient certainty the equal brightness. Reliable and rapid measurements can be made on film not less than 100 Å thick.

**Electron microscopy.**

An AEI microscope was used with a high voltage of 80 kV and double condenser, 400 μ diaphragm in the condenser and 150 μ in the objective lens, magnification 7,500 x. Alternatively a Siemens Elmiskop IA was used, with a high voltage 80 kV, a single condenser (K, unexcited), 200 μ diaphragm in the condenser, 50 μ in the objective lens, magnification 8,000 x, following the technique introduced in our Laboratory by A. K. Kleinschmidt. The advantage to use the microscope at very low magnification seems to be due to the very low intensity of illumination requested in this condition. In fact, to reach at 80,000 x the same intensity of illumination as at 8,000 x, a beam current one hundred times higher is necessary. That means a diffuse background intensity and a contamination rate proportionally higher.

The image were recorded on Kodak Electron Image plates, which were developed by undiluted D 19 in 2 min.

**RESULTS**

It was possible to obtain a velocity of sedimentation of the carbon film which remained constant for many hours (Fig. 2), by ensuring the stability

![Fig. 2. — Thicknesses of carbon film in Å versus evaporation times in hours: the velocity of sedimentation remains constant for many hours.](image-url)
of the carbon evaporation procedure. This was achieved by standardizing the geometry of the system, thoroughly cleaning the work chamber, accurately outgassing before the evaporation process, achieving a good vacuum (2 \times 10^{-5} \text{ torr}) during evaporation, and continuously controlling the current in the electron gun.

As can be seen from Fig. 2, the time versus thickness relationship is represented by a straight line, which passes through the origin, so that it can be assumed that the relationship remains linear until extremely thin films are obtained, even if the direct measurement of the thickness of the film becomes uncertain below 100 Å.

If the work chamber is dismantled, all the pieces cleaned and the carbon changed in the electron gun, the derivative of time–thickness function may change as much as 10–20 %; so that an error of this magnitude must be taken into consideration in the estimation by this method of the thickness from the evaporation time.

It may be observed from Fig. 2 that the time required, for instance, to reach a thickness of 100 Å by this device is much longer than the time required in the usual shadowing apparatus (2h 30 min against few seconds); but it must be considered that, to obtain ultrathin films, this time is proportionally reduced and in any case the unpredictable time required to measure and select very thin films obtained with conventional methods is completely eliminated (see, for instance, the laborious measurement technique employed by Whiting & Ottensmeyer, 1972).

On the basis of these results, attempts have been made to evaluate the minimum thickness of a carbon film consistent with the mechanical stability needed for use as a supporting film in electron microscopy. It has been seen that down to 10–15 Å the film can be manipulated, but below this thickness the film seems to disintegrate when detached from the supporting surface, floating on water. This finding is in agreement with some data obtained by Hayek & Schwabe (1970): according to these AA., the carbon film evaporated at temperature between 25 and 350 °C grows from an island stage to a porous structure, and reaches the coalescence stage at a thickness between 10 and 15 Å when evaporated at 260 °C. In our case the temperature of the supporting surface during evaporation rose from about 50 °C (after the outgassing) to about 100 °C for ultrathin films, reaching 123 °C for 75 Å thin films and remaining at this value for longer evaporation times.

Images of unstained biological specimens on 15 Å carbon films are shown in Plates 1a, b, c. In Plate 1a, 50s ribosomes can be seen. The specimen was obtained by Araco et al. (1971) from E. coli, buffered with 10^{-3} \text{ M tris-HCl} with 10^{-3} \text{ M Mg acetate}. The aspects of the unstained ribosome units visible in the picture are in keeping with those observed by Spirin et al.
(1963) in a stained specimen. Preparations from a solution of tropocollagen molecules are seen in Plate 1b and c. Finely divided collagen from a rabbit tendon was treated with 0.5 p. c. acetic acid for 24 hours and purified by precipitation with 5 p. c. NaCl solution according to Cox et al. (1967). Plate 1b shows the unstained specimen; it appears as an irregular network made up of strands 30-40 Å thick and 300-3000 Å long, which could correspond to the «Fast-sedimenting component » obtained by BÉRÉR & ENGEL (1966) at low temperature. In Plate 1c the same preparation, negatively stained with phosphotungstic acid (pH 7.4), is seen. Long filament, up to 1000 Å in thickness, are observed, which are never visible in the unstained material. This observation supports the view of OLSEN (1965) on the possible packing action of phosphotungstic acid on tropocollagen molecules, when used as a negative stain.

CONCLUSIONS

The method outlined in this paper enables carbon films of known thickness to be made with an accuracy of 10-20 %, having a high mechanical stability, up to approximately 15 Å in thickness. As the aim of this work is to obtain extremely thin supporting films for electron microscopy, the precision in the determination of the thickness is unessential: it is important, on the contrary, to establish a method with which it is possible to fix in advance the thickness of the supporting film depending on the size and type of the specimen under study. Such a condition is irrelevant when negatively or positively stained specimens are observed; but it is of fundamental importance when unstained material is under observation.

The results obtained in pictures as those shown in Plate 1a, b, c confirm this assertion: to our knowledge, it is the first time that ribosome subunits or tropocollagen molecules have been seen with such a good contrast and resolution without staining.

As can be seen from a comparison of Plate 1c with Plate 1b, an unstained specimen may give an important indication on the packing arrangement of macromolecular units in the absence of environmental factors, other than the adhesion to the supporting film.

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REFERENCES


Plate 1. — a — Unstained 50s ribosome units from E. coli, on 15 Å carbon film. (x 184,000).
b — Unstained tropocollagen molecules from a rabbit tendon, on 15 Å carbon film. (x 37,000).
c — The same specimen shown in 1b, but negatively stained with phosphotungstic acid, pH 7.4. (x 77,000).
Ultrastructure of native collagen

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Summary. – Native collagen fibrils, mechanically detached either from leg tendon of rabbits or from tail tendon or aortic adventitia of adult rats were observed at the electron microscope by various technical methods.

The density patterns recorded along and across integer fibrils, laid down on ultrathin C films, show that the density ratio of the light to the dense bands is much higher than that expected from the generally accepted quarter-stagger arrangement of collagen unities.

Swollen fibrils show long filaments, about 50 Å thick, reasonably identified with their building unities. The characteristic collagen banding of about 640 Å periodicity, never exhibited by single filaments, is already present in groups of very few filaments (order of two or three). Partially disrupted fibrils suggest that the building filaments may be assembled in a monolayer, having the form of a long ribbon, spirally wound around the longitudinal axis of the integer fibril.

Riassunto (Ultrastruttura del collagene nativo). – Fibrille collagene native, distaccate meccanicamente dal tendine della zampa del coniglio oppure dal tendine della coda o dall’adventitia aortica del ratto adulto, sono state osservate al microscopio elettronico impiegando vari metodi di preparazione.

Le densitometriche fatte lungo fibrille integrate o trasversalmente ad esse, (essendo queste deposte su film di C ultrasottili) mostrano che il rapporto tra la densità delle bande chiare e quella delle bande scure è assai più elevato di quello richiesto dal comunemente accettato modello (quarter-stagger array) della disposizione degli elementi unitari del collagene nella fibrilla.

Nelle fibrille rigonfie si osservano lunghi filamenti aventi circa 50 Å di spessore, che sono da ritenersi gli elementi costitutivi della fibrilla. La periodicità caratteristica del collagene, di circa 640 Å, non si osserva mai in questi filamenti, ma è già osservabile in gruppi di due o tre di essi. L’osservazione di fibrille parzialmente disfatte fa supporre che questi filamenti elementari si aggreghino in monostrato, e che questo sia avvolto a spirale attorno all’asse longitudinale della fibrilla.

INTRODUCTION

Collagen fibrils are mainly formed by a protein, tropocollagen, which has a molecular weight of about 300,000. From X-ray diffraction data (Ramachandran, 1967) it is known that this molecule is made up to 3 helical polypeptid chains wrapped in a right handed super-helix, the whole molecule being formed by about 14 Å in cross section. The fibrils are para-crystalline arrays of these long chains. The arrangement gives a periodical structure which shows up clearly at the electron microscope, both in positive and negative stain.

A large amount of work was involved in understanding how the tropocollagen (TC) molecules are packed in order to produce this periodical structure, given the important role that collagen has in connective tissue properties and disorders. All the same the problem is still open. The limitations in our knowledge of fibrillar structure reflect technical restriction. In fact, low angle X-ray diffraction technique gives a poor resolution because of the inexact packing lattice of collagen; physico-chemical data are not sufficient to settle a model of the packing array, so that the most striking results were obtained from electron microscopy. But by this technique the direct investigation of native unstained collagen fibrils under the electron microscope is prevented by the low contrast given by biological material. Consequently electron microscope data are based on studies and discussions of images of collagenous material in negative and positive stain. But the interaction of tropocollagen molecules with the staining salts cannot be disregarded, as it may be seen (Steve Bocciarelli & Tangucci, 1974) that this material can undergo different aggregation states if observed with or without negative staining.

With electron microscopy a comparison was made between the images of native collagen fibrils and those of artificial aggregates, the SLS or segment-long-spacing which have been thought to be a parallel array of the TC molecules polarized in the same direction and with regions of corresponding chemical properties in register (Hodge & Smith, 1960; Olsen, 1963). SLS frequently were formed adjacent to reconstituted collagen fibrils, spanning 4 periods of the fibrils in a reproducible position, and this observation led to the conclusion that TC molecules are packed in native collagen fibrils following a «quarter-stagger» array. Later on, this model was modified after a comparison of the length of SLS and the length of another artificial aggregate, the FLS or fibrous-long-spacing. This was considered to be a parallel array of TC dimers made of molecules attached end-to-end. This comparison showed that in native fibrils the molecules are not attached just end-to-end, but with the superimposition of about 1/10 of their

length. In conclusion, the final pattern of the structure of native fibrils seemed to be that of Fig. 1. Unfortunately some difficulties arose: this type of packing produces a monolayer sheet, but it is geometrically impossible in three dimensions (McGravin, 1964). This difficulty may be overcome if a random choice in combining the bonding zones of the macromolecules to form the native collagen fibrils is accepted (Cox et al., 1967).

A second difficulty is that the threadlike structures or filaments composing the collagen fibrils seem generally too thick to be considered linear polymers of individual TC molecules. In fact, according to Olsen (1963), they are 15–30 Å in diameter and it is assumed that they represent from one to four TC molecules. The same is true of the filaments forming the SLS or FLS. These dimensions are in agreement with the data obtained from solubilisation experiments, which suggest that the filaments could be built from 4 or 5 molecules. These may be supposed to be assembled by the quarter–stagger method and have a helical arrangement (Veis et al., 1967). Miller & Wray (1971) performed a detailed analysis of the information which they could obtain with the equatorial X-ray diffraction pattern of collagen lattice and they concluded that the filament is a five — stranded rope («coiled coiled coil»), but they have not defined the lattice of filaments in native fibrils. Smith (1968) reached the same conclusion on different experimental grounds and Segrest & Cunningham (1973) after indirect analytical approaches.

If it is accepted that collagen fibrils are not made by single TC molecules but by thin «ropes» of 4 or 5 of them, the problem is again open of how the fibrils are built from these ropes so as to have the well known periodicity of about 640 Å.

Fig. 1. — Diagram of the quarter–stagger arrangement of collagen monomer units.
In 1971 Pease & Bouteille, applying the method of "inert dehydration", observed sections of unfixed collagen from rat aortic adventitia and showed that the individual filaments, that is the structural unities of the fiber, are arranged in a helical pattern. The same result was recently obtained by Rayns (1974) after having applied the freeze facturing technique to cardiac collagen: he found, moreover, that this filament array is consistently right handed.

Nor must the presence of carbohydrates in or around the fibers be disregarded, as they seem to be bound to the collagen, even if their amount is estimated to be very low in vertebrate, of the order of 1% (Eastoe, 1967).

By means of ruthenium red selective staining Myers et al. (1969) found the following different types of red-positive structures associated with synovial collagen: 1) amorphous coats surrounding individual fibrils; 2) transverse belts overlying the major periodic bands; 3) fine lateral filaments extending outwards from the fibrils; 4) intermediate fibrils intermingled with collagen fibrils. Pease & Bouteille (1971), using the acid PTA stain, gave evidence of carbohydrate, in an amorphous space-filling matrix which is placed between discrete filaments, all along the fibrils.

Both the finding of Myers et al. (1969) and those of Pease & Bouteille (1971) show amounts of carbohydrates which seem to be too high to be in agreement with biochemical estimates, but this discrepancy may be explained (Pease & Bouteille, 1971) by a very favourable ratio between the amount of staining and that of stained material.

In our laboratory a method has been developed for the preparation of ultrathin carbon films (up to 15-20 Å) which are continuous and strong enough to be employed as supporting films (Steve Bocciarelli & Tangucci, 1974). Their use gives the possibility of reaching a comparatively strong contrast in the images of unstained biological material, in both clear and dark field microscopy. With this technique density patterns performed on the electron microscope images acquire significance, as the thickness of the supporting film is negligible in respect to that of the specimen, which, in the present case, is of the order of 1000 Å. Moreover, partially disrupted fibrils may be observed without any interaction with staining salts. Even if the images are not equally brilliant, some results may be obtained which cannot be reached by the use of staining method.

This report is concerned with the contribution made by the results obtained in our laboratory towards solving the problem of the assembling of native collagen fibers. These results have been briefly reported elsewhere (Steve Bocciarelli, 1974).
MATERIALS AND METHODS

a) Suspension of native collagen fibrils.

Bundles of native collagen were excised from leg tendon of rabbits or from tail tendon or aortic adventitia of adult rats. The drafts were cut into small pieces and collagen fibrils were swiftly separated by mechanical teasing, in bidistilled water.

b) Partially disrupted fibrils.

A solution of 0.05 p.c. acetic acid in bidistilled water was prepared, a small quantity of the suspension of fibrils from rabbit tendon, in water, was placed on a slide and covered with a drop of the acetic acid solution for a few minutes (1–5). Collagen from rat tail tendon was found partially swollen after the mechanical teasing in bidistilled water, and a solution 0.005 p.c. was needed to obtain a preparation showing fibrils almost all partially swollen.

c) Solution of tropocollagen.

The suspension of collagen fibrils in water was dialyzed for 24 h in 1 p.c. ammonium acetate at 4 °C. To the dialyzed suspension an equal volume of 0.4 p.c. acetic acid was added and the mixture was left 24 h at 4 °C. The mixture was filtered through a micropore glass filter 100–200 if too viscous, the solution was diluted with 0.2 p.c. acetic acid, in order to accelerate the procedure. The filtrate was purified by precipitation with NaCl solution (5 p.c. final NaCl concentration). The precipitate was centrifuged 15 min at 1000 rpm and redissolved in bidistilled water. The suspension was again dialyzed, an equal volume of 0.1 p.c. acetic acid was added, and the mixture was left 24 h at 4 °C.

d) Thin section of collagen.

Fresh tissues were immersed in 1 p.c. OsO₄ in acetate veronal buffer pH 7.4 (Michaelis buffer) for 1 h, dehydrated in ethyl alcohol series and embedded in Araldite. Sections were stained with 1 p.c. PTA or 2 p.c. uranyl acetate or else with double staining (uranyl acetate and lead citrate).

e) Freeze etching.

Fresh tissues were immersed in 20 p.c. cold glycerol for 30 min, frozen in liquid Freon 22 and transferred in the Balzer freeze–etching apparatus. Sections were obtained at −100 °C and sublimation time was 1 min 30 sec. The replicas were cleaned in sodium hypochloride for 2 h and washed in numerous changes of water.

f) Negative staining.

A solution of 2 p.c. phototungstic acid in bidistilled water, which had been adjusted to pH 7.3 or 7.4 by the addition of KOH (Brenner & Horne, 1959) was used as a negative stain.
g) Ultrathin supporting films.

Amorphous carbon was evaporated on freshly cleaved mica sheet, in a modified Edward shadowing apparatus, as previously described (Steve Bocciarelli & Tangucci, 1974). Carbon strata 15–25 Å thick were used as supporting films for clear and dark field microscopy of unstained specimens.

h) Electron microscopy techniques.

As the supporting film is very fragile it is impossible to put a drop of the suspension to be examined on the coated grid, using a micropipette. To preserve the carbon film it is preferable to pass the coated grid gently, face down, over the surface of a drop of the suspension, and leave it to dry. If a negative staining is wanted, the suspension must be mixed with the stain and the same procedure may be followed, but usually an ultrathin supporting film is not used in this case. Sometimes however a sort of autonegative staining of the specimen is found, as small quantities of residual fats or other material may be sufficient to act as such a stain, given the extreme thinness and transparency of the supporting film and of the specimen itself.

A Siemens 102 electron microscope was used, with a high voltage 80 kV and double condenser, 200 diaphragm in the condenser and 50 in the objective lens. Photographs were taken usually at magnification 8000 x, to reduce the background intensity and the contamination (Steve Bocciarelli & Tangucci, 1974), sometimes at 40,000 x, when stained specimens were observed.

Dark field microscopy was performed by the source tilting method.

i) Density patterns.

A Joyce-Loebl densitometer was used and density patterns along and across integer fibrils were recorded.

RESULTS

a) Integer fibrils in clear field microscopy.

Fibrils isolated from leg tendon of rabbits or from tail tendon of rats were observed by transparency and when they appeared clean and with a neat banding, images like that of Fig. 2 were obtained. The pictures were processed at a Joyce Loebl densitometer and density patterns along and across the fibril axis were recorded. Since the supporting carbon film, 10–25 Å in thickness, makes no appreciable contribution to the density of the image, in objects which, like these fibrils, are no less than 2000 Å in thickness, there is no doubt that the density patterns so obtained are a recording of the density distribution in the fibrils.
Both in fibrils isolated from rabbit tendon and in those from rat tendon the density ratio of the light to the dense bands as measured in the central zone of bands, was, as a mean, a little higher than 90%. This result has to be compared with that given by the quarter-stagger model of the tropocollagen assembling of the collagen fibrils: from Fig. 3 a ratio of 80% is easily evaluated.

The difference cannot be ascribed to experimental errors in the measurements of the density ratios, because if a density pattern across the fibrils was recorded along both a clear and a dark band, the right distribution of density to be expected from a cylindrical homogeneous structure was found; moreover, the density value of 80% in respect to that recorded at the center of the dark bands was properly found, in the same bands, at a distance from the axis which is 60% of the radius of the fibrils cross section: this value was directly compared, as a control, with the density at the center of light bands.

The excessive value of the density ratio of the light to the dense bands could be due to a retraction of the fibrils during the dehydration on the carbon film, or to a contribution in density coming from a material surrounding the fibrils or embedding them. The first hypothesis may be discarded because the banding in the fibrils appears to correspond to the well known periodical array of about 640 Å, with no alteration in the length of the light bands in respect to the dark ones. If the second hypothesis is right, an envelope, made for instance of carbohydrates, surrounds the fibrils or at least the light bands, or else embeds them. If an envelope surrounds the whole fibril it is easy to show that if it may change the density ratio of the light to the dense bands from 80% to more than 90%, it has to be about of the same thickness as the fiber itself, if its density is about the same.

As a matter of fact, fibrils surrounded by an amorphous coat were frequently observed, and they were clearly evident in dark field microscopy (Fig. 3). Moreover the presence of a carbohydrate envelope around the whole fibril has been recognized by many AA. Acid mucopolisaccharides have been demonstrated in sinovial collagen by Myers et al. (1969), and in various connective tissues by Kajikawa et al. (1970). Bouteille & Pease (1971) identified with carbohydrates the thin peripheral zone of collagen which in the sections of aortic adventitia embedded after «inert dehydration» appears emphasized by the acidic PTA staining. Similar results may be obtained in osmium fixed fibrils from rabbit tendon, after neutral PTA staining, as shown in Fig. 4; even with the freeze-etching technique such an envelope is observed around the fibrils (Fig. 5a and Fig. 5b).

The thickness of these envelopes seems to be no more than 1/10 of the cross section of the fibrils, and equally distributed along the fibril, so that a significative contribution of the envelope in determining the density
ratio from light to dense bands seems to be excluded. All the same, experiments were performed to remove carbohydrates: collagen fibrils were treated with hyaluronidase in phosphate buffer pH 5.4 for 4 h, washed in the same buffer, and density patterns were obtained from the fibril pictures. The density ratio was evaluated as described above, and the same value, that is more than 90%, was obtained.

b) The building units of the fibrils.

After a mild treatment in a week solution of acetic acid, the fibrils are swollen and their structural units appear as long thin filaments. These filaments may be separated without complete disruption of the basic space all along the fibrils, as shown in Fig. 6 with negative staining. Similar results have been obtained by Bairati et al. (1970) in collagen fibrils from human dermis.

Measurements performed on these filaments, in images obtained in negative staining, show that they are from 35 to 50 Å thick. Their length is indefinite, but it seems to span more than 4 or 5 collagen bands. Rarely the end of a filament is seen in partially disrupted fibrils; if this happens, a small knob about 70 Å in diameter may be seen attached to it.

Filaments seem to be flexible and are never smooth: fine granules (about 50 Å in diameter) are usually seen along them. Pictures obtained from unstained specimens (Fig. 7) or in autonegative contrast (as explained in 2, b) (Fig. 8) confirm these results.

It could be supposed that the minimum thickness of about 35 Å measured in these pictures could be given by the technique itself. To check this point TC molecules were prepared and observed without any staining, both in clear field as the previous specimens, and in dark field microscopy. Fig. 8 shows tropocollagen molecules in clear field microscopy. The contrast given by single molecules is extremely poor because the thickness of the specimen (15 Å) is just a little less than that of the supporting film (20 Å). In this condition the physical characteristics of the photographic emulsion make the thickness measurements worthless. The results in dark field microscopy are no more satisfactory. All the same in images like Fig. 9 tropocollagen molecules appear so faint in comparison to the filaments seen by the same technique, for instance in Fig. 7 that we are bound to admit a difference in thickness between the two structures.

We may conclude that the filaments are made by the lateral aggregation of no less than three tropocollagen molecules. If we consider that 50 Å is a very frequent diameter of the filaments, the numbers 4 or 5 seem equally probable.

These filaments appear to be the building unities of the fibrils. They could correspond to the tetramers postulated by Veis et al. (1967) which
are supposed to be a right handed helical arrangement of four tropocollagen molecules displaced successively so as to preserve the quarter-stagger ordering of Fig. 3, or else the pentamers suggested by Smith (1968). The beaded appearance could be in agreement with a helical arrangement, but a periodicity of about 70 Å seems too high a value to correspond to the helical arrangement of four of five wound elongated molecules about 14 Å thick. On the other hand, collagen banding of 640 Å periodicity, which may be recognizable even in small groups of filaments, never appear (Fig. 6), in single filaments so that we have no data to confirm the hypothesis of a quarter-stagger arrangement of tropocollagen molecules in these structures.

c) Packing arrangement of the filaments in native fibrils.

In negatively stained swollen fibrils, as already observed, single filaments never show the banding which is supposed to be the basis of the periodicity of about 640 Å found in the native collagen fibrils. When the picture shows the beginning of the disruption of a band, an attempt may be made to understand how the filaments are linked to each other to make up a band. It appears that the banding is preserved even if groups of very few filaments are left, may be two or three. The lateral connection between the filaments seems to be made without interrupting or elsewise perturbing the course of the filaments themselves: small knobs (about 70 Å in diameter) seem to be the binding structures, and they appear in a row on the borderline of the clear band. Granules of the same dimension may be found apparently free in swollen fibers, or at the end of detached filaments.

Pictures obtained without any staining, from partially disrupted fibrils laid down on ultrathin carbon films, obviously give a poorer contrast in the image. But in images of this type the density recording of the object is reported, so that a relief effect may result. From some pictures (Figg. 10 and 11) portions of the fibers appear which suggest that the filaments may be assembled in a monolayer having the form of a long ribbon wound in a spiral around the longitudinal axis of the fibrils. In these pictures zones in which the original lattice of the fibril is preserved are alternated with zones in which the monolayer is spread out. This effect may be due to the adhesion of these last zones to the supporting film, aided by the action of the water surface tension during the drying of the specimen. The periodicity of 640 Å of the native fibrils is clearly evident not only in the rolled up portions but even in those which are stretched out. This banding seems to be inclined a few degrees (no more than 5° or 6°) in respect to transversal axis of the monolayer: this condition is geometrically important because only a band having an inclined periodical banding is able, after rolling up into a tight helix, to produce an elongated structure having a transversal periodicity, that is a structure similar to the lattice of native collagen fibrils.
DISCUSSION

From the experimental results reported in this paper evidence seems to be given that collagen fibrils can be formed by filaments 35-50 Å thick attaching themselves to one another laterally in the form of a long narrow sheet, which then rolls up to form a helical structure.

A structure of this type was predicted by Ramachandran and Sasi-sekharan as an explanation of a series of peaks obtained in small angle diffraction patterns by Cowan and Coll. from wet specimens of tendon (Ramachandran, 1967). These AA. reached the conclusion of the occurrence of a cylindrical lattice with a finite number of sheets, or a roll structure. The elementary unit of this structure was the triple-helix of tropocollagen molecule. The whole aggregate in natural tendon had a diameter of approximate 200 Å: it was named «cryptofibril». Miller & Wray (1971) on a similar experimental basis seemed to confirm these results. On the contrary, at the electron microscope it is unusual to see in partially disrupted fibrils elongated structures about 200 Å in diameter and showing the characteristic 640 Å axial period as requested by Ramachandran and Sasi-sekharan assumption. When the fibril begins to be dismantled (Fig. 12), the more frequent aggregate which appears and is maintained in more advanced stages (Fig. 6) is a packing of no more than 4-5 tropocollagen molecules. On the other hand from low angle diffraction experiments Miller & Wray (1971) deduced that a lateral packing of tropocollagen in a five-stranded rope, as suggested by Smith (1968), was consistent with their results.

A helical winding of filamentous substructures in collagen fibrils seems to be confirmed by other experimental evidences. Bouetelle & Pease (1971) in unfixed collagen from aorta adventitia of the rat, «inherently dehydrated», embedded in hydroxypropylmetacrilate, sectioned and stained with uranyl acetate and or lead citrate, observed filamentous structures 30-35 Å thick, arranged in a helical pattern. These AA. affirm that the fibrils prepared by this technique were «expanded», so that a clear visualization of fibrillar filament could be achieved.

We have tried to observe collagen from aortic adventitia of the rat with the freeze-etching technique and have obtained (Fig. 5 b) some images confirming the existence of spiralized filaments. On the contrary, fibrils from rabbit tendon which, in our experience, are more resistant to chemical agents than aortic adventitia or tail tendon of rat, never show a spiralized course in the filaments in glicerinated freeze-etched specimens (Fig. 5 a).

We may conclude that filaments arranged in a helical pattern within fibrils are seen only when the whole structure is altered in a suitable way. If we consider the experimental techniques used to prepare the specimens, we may suppose that the results obtained by Rayns (1974) and quite
recently by Belton et al. are in agreement with this assumption. But if the spiralized filaments appear in unrolling structures, in these structures the 640 Å banding has to be found inclined in respect to the longitudinal axis of the fibrils, as the filaments seem to be generally normal to the cross banding of the fibrils. The effect, actually, may be observed in the pictures of inherent dehydrated fibrils published by Boutelle & Pease (1971): the 640 Å banding seem to be inclined to the transverse plane at a few degrees. On the contrary, when the native fibrils are integer, so that the monolayer is tightly rolled up, even in aortic adventitia collagen the banding appears, normal to the longitudinal axis of the fibril, as it may be seen in Fig. 13 in negative staining.

The problem of the packing of tropocollagen to form the filaments or «ropes» seems moreover to be worthy of discussion: from small angle diffraction patterns the resolution required to solve this problem cannot be attained, and after morphological observations the quarter-stagger assembling of the «ropes» from tropocollagen molecules seems not to be confirmed. The 640 Å banding, indeed, never is observed in single filaments, but it may be observed when two or three filaments are bound together. If this is so, we have to assume that peculiar lateral bindings are present and we could identify them with the small spots which are clearly evident in partial disrupted fibrils (Fig. 6).

If the quarter-stagger model cannot be supported either by morphological evidence on single filaments, or by density measurements along integer fibrils, or by X-ray diffraction patterns, we may ask if it is really necessary: lateral bonds acting between filaments may justify the higher density measured in the so-called dense bands in comparison with the others, and the proposal of the quarter-stagger assembling of TC molecules, which was made to explain just why this axially repeating band pattern may be discarded.

CONCLUSIONS

The experimental data reported in this paper, compared with those obtained by others, may be explained if the following model for the native collagen fibril is accepted: the triple helix tropocollagen molecules are assembled in filaments made up of the lateral packing of 4–5 molecules. These filaments are assembled in a monolayer, a long narrow ribbon showing the 640 Å banding characteristics of collagen fibrils. The monolayer is rolled up to form a helical structure which appears to be loose when the fibril is swollen, but is tightly wound when the fibril is integer.

The hypothesis of the quarter-stagger array of TC molecules is unnecessary and seems not to have experimental confirmation on the basis of morphological and X-ray diffraction pattern data.

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Fig. 2. — Unstained fibril, from rabbit tendon, observed by trasparency in clear field microscopy.
Fig. 3. — A partially disrupted fibril in dark field microscopy: an amorphous coat is observed around the integer portion of the fibril.
Fig. 4. — Section of osmium fixed fibrils from rabbit tendon, stained with PTA, pH 7.4.
Fig. 5a. — Fresh tissue from rabbit tendon, prepared with the freeze-etching technique. An amorphous coat seems to surround the fibrils.

Fig. 5b. — Fresh tissue from aortic adventitia of adult rats, prepared with the freeze-etching technique. A spiralized course in the filaments is seen.
Fig. 6. — Swollen fibrils from rabbit tendon, negatively stained with PTA, pH 7.3. Rough filaments 35–50 Å thick and of indefinite length seem to be the structural units of the fibrils.
Fig. 7. — Unstained swollen fibril from rabbit tendon. The same structural units as in Fig. 7 are seen.
Fig. 8. — A swollen fibril in autonegative staining, that is with a background given by the specimen itself.
Fig. 9. — Unstained tropocollagen molecules as seen by transparency in clear field microscopy. The molecules, about 15 Å in diameter are on a supporting film about 20 Å in thickness.
Fig. 10. — Unstained partially disrupted fibril, clear field microscopy.
Fig. 11. — Unstained swollen fibril, clear field microscopy.
Fig. 12. — A fibril at the very beginning of the swelling process.
Fig. 13. — Negatively stained fibril, from aortic adventitia of rat.