

ISOLATION OF MICROFIBRILS FROM RAT VIBRISAL FOLLICLES

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The fibrillar nature of wool keratin is well established but considerable doubt still exists concerning the mode of aggregation of the constituent protein molecules.

Early workers (7, 10, 12, 22) showed that it is possible to examine the contents of cortical cells of suitably treated wool samples with the electron microscope, and that (macro)fibrils, which can be discerned in the optical microscope (10), consist of smaller filaments with diameter ranging from 500 nm down to about 10 nm. Farrant et al. (7) considered the microfibril to consist of globular keratin molecules (10 nm in diameter) but it was pointed out by Jeffrey et al. (12) that "sufficiently drastic treatments (of wool) are effective in producing a globular appearance in the microfibrils" and they observed that the more complete isolation of microfibrillar material required extreme breakdown of both hydrogen bonds and disulphide cross-linkages. However, with the advent of ultramicrotomy and the application of electron stains such as osmium tetroxide (1, 19), thin cross-sections of wool revealed the typical filament-matrix texture, which consists of microfibrils, 7-8 nm in diameter, embedded in an osmiophilic matrix (19). High resolution micrographs show microfibrils to possess a fine structure which Filshie and Rogers

(8) considered to represent a "9 + 2" arrangement of protofibrils but, due to the phase structure inherent in such images, precise deductions are difficult and no firm conclusions can be drawn (13, 18). Later work on the disruption of reduced wool (5, 6) and also unmodified hair follicle (21) by ultrasonic irradiation in formic acid revealed, with negative staining, sheets of fibrillar material and also individual filaments between 2 and 3 nm in diameter. Dobb and Rogers (6) suggested that these filaments aggregated in the "9 + 2" arrangement to form the microfibril in the intact fiber. Individual microfibrils, with a diameter of 7-8 nm, were not observed in these preparations. Doubt was cast upon the conclusions drawn from the results obtained with ultrasonic degradation of chemically modified wool in a recent communication to this journal (17), in which Millward indicated that the protofibrils could in fact be due to cellulose contamination.

The present investigation is concerned with the reexamination of the problem of the isolation of fibrillar material from keratin, avoiding the use of drastic chemical and physical treatments. Since most of the effort required to disperse keratinized fibers has, in the past, been directed at breaking down the high degree of disulphide cross-linking,

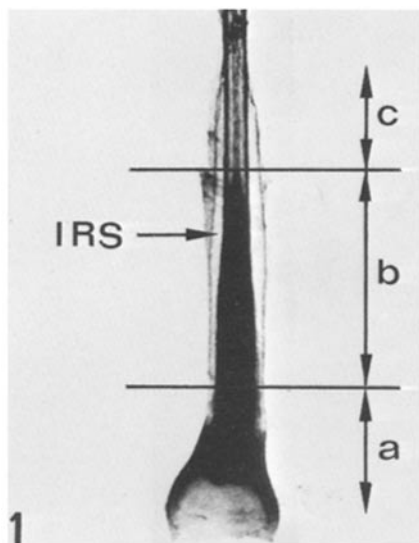


FIGURE 1 Rat vibrissal follicle immersed in phosphate buffer showing levels at which dissection was made. (a) Lower bulb region; (b) keratogenous zone, used for preparing dispersed materials; (c) keratinized shaft. *IRS*, inner root sheath.

it seemed relevant to attempt isolation of fibrillar material from the follicle before the disulphide cross-linkages are formed and the protein is still in a relatively unstable form (1, 16). Rogers (20) has examined proteins extracted from wool roots and has observed microfibrillar sheets in aqueous homogenates but was in some doubt as to the exact origin of this material, mainly because of the presence of inner-root sheath cells. For the purpose of examination of the follicle material by electron microscopy, this latter problem has been overcome by dissecting small quantities of follicles and separating the relevant components by suitable chemical treatments.

MATERIALS AND METHODS

Rat vibrissal follicles, chosen for their convenient size, were plucked from freshly killed or anesthetized rats and immersed in either distilled water, 0.1 M phosphate buffer, pH 6.5, or fixed in a solution of 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 2 hr.

Dispersed material was prepared in the following manner. With the aid of a binocular microscope, anagen follicles were stripped of their inner-root sheath cells while immersed in water or buffer solution and dissected in the proportions indicated in Fig. 1. The middle portions (b) in Fig. 1, containing both prekeratin and keratinized material (16), from

six to ten follicles, were transferred directly to 1 ml of a freshly prepared solution of 0.1% chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.) in 0.1 M phosphate buffer, pH 6.5, at room temperature, and agitated gently with a glass rod for varying periods of time between 10 min and 1 hr. The resulting dispersion was centrifuged for 10 min at 2000 g , washed several times with buffer solution followed by distilled water, and finally redispersed in 2 ml of distilled water. Small quantities of the freshly prepared dispersion were spread on to holey carbon films supported on copper grids (3.05 mm) and treated with one drop of either 1% sodium phosphotungstate (PTA), 2% uranyl acetate, or 1% uranyl formate solution, the excess being withdrawn after 2, 10, and 15 min, respectively. It should be noted that considerable care was taken with the present work to exclude bacterial contamination, by using freshly distilled water and previously boiling the glass apparatus employed. Bacterial growth was discouraged in specimens stored overnight in the refrigerator, by the addition of a small quantity of chloroform or toluene.

Follicles fixed in the glutaraldehyde solution were washed in buffer solution containing 7% sucrose and immersed for 30 min in a solution of 1% osmium tetroxide in Veronal-acetate buffer, pH 7.0. After dehydration through graded alcohol-water mixtures, the follicles were embedded in Araldite resin and sections were cut on an LKB ultramicrotome. Thin sections were treated with lead citrate solution (3).

Specimens were examined in a JEM 100BII electron microscope operating at 100 kv with a 40 μ objective aperture, and images were recorded on Agfa 23D 56 cut film. The microscope magnification was calibrated, for each different specimen cartridge, using the (20I) lattice planes of platinum phthalocyanine (1.194 nm).

RESULTS

A good yield of well dispersed cortical material was obtained after short times of treatment, usually 30 min followed by 10 min centrifugation, and only gentle agitation in the enzyme solution was necessary. Macrofibrils, about 240 nm wide on the average, similar to those evident in the longitudinal thin section shown in Fig. 2a, are easily identified (although lacking the fine fibrillar structure) and can be seen to fray at their extremities into many smaller filaments (Fig. 2b) with uniform diameters of 7.2 nm (40 measurements with standard deviation, $\sigma = 0.2$ nm) and of considerable length (Fig. 3a).

Samples prepared by treating follicle material with the enzyme solution either for extended periods of time (greater than 1 hr) or at slightly

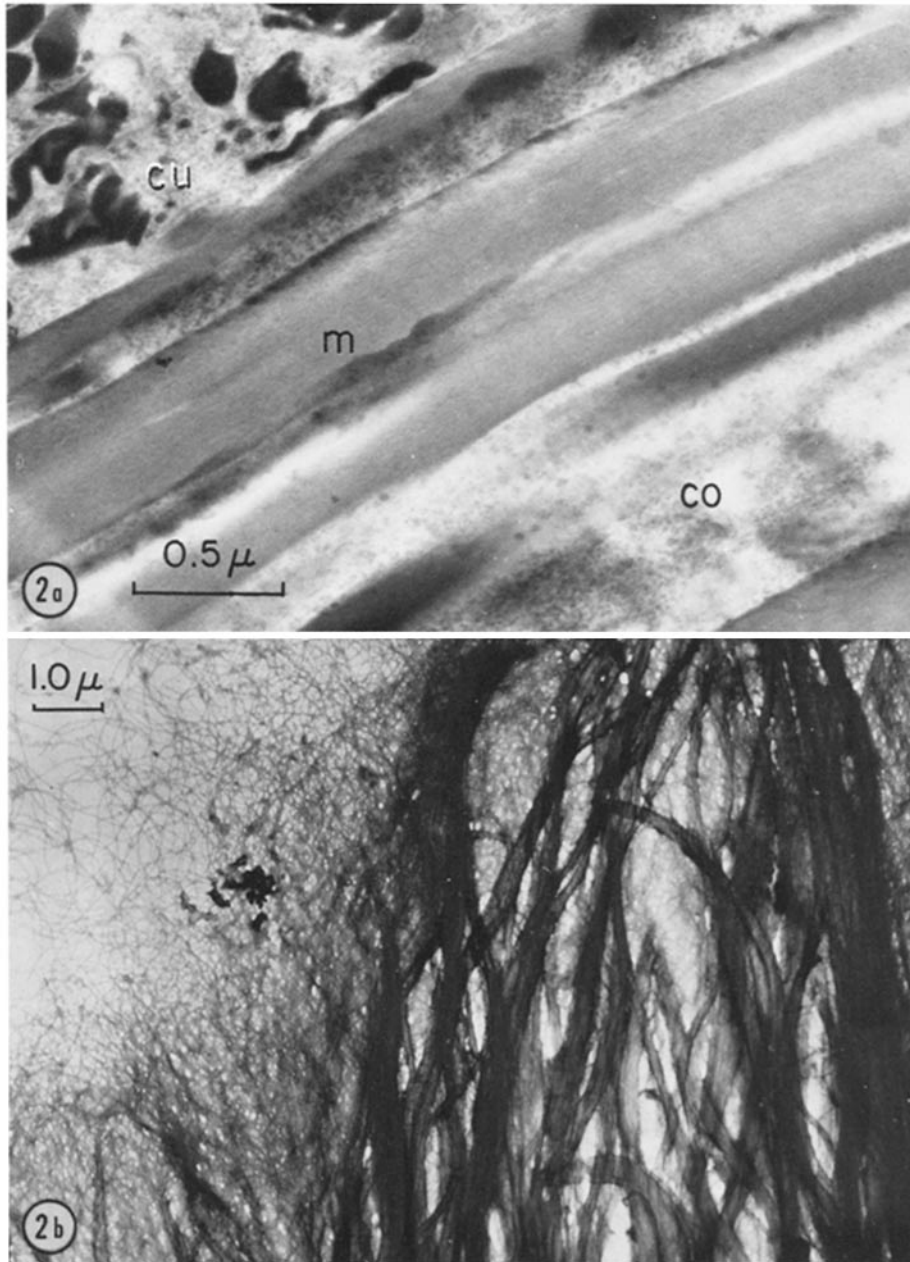


FIGURE 2a Longitudinal section of rat vibrissal follicle in approximate region of Fig. 1(b). Note the fibrillar structure revealed in the macrofibrils (*m*). *cu*, cuticle; *co*, cortex. Lead citrate. $\times 40,000$.

FIGURE 2b Low magnification image of dispersed cortical material from rat vibrissal follicles treated with 0.1% chymotrypsin. PTA, pH 3.0. $\times 9300$.

elevated temperatures (40°C) did not clearly show the fine subdivision of the macrofibrils, probably due to excessive degradation of microfibrillar material. Shorter times of treatment, 5–10 min,

gave insufficient separation of the microfibrils. They appeared less well defined and were apparently contaminated with other cellular material.

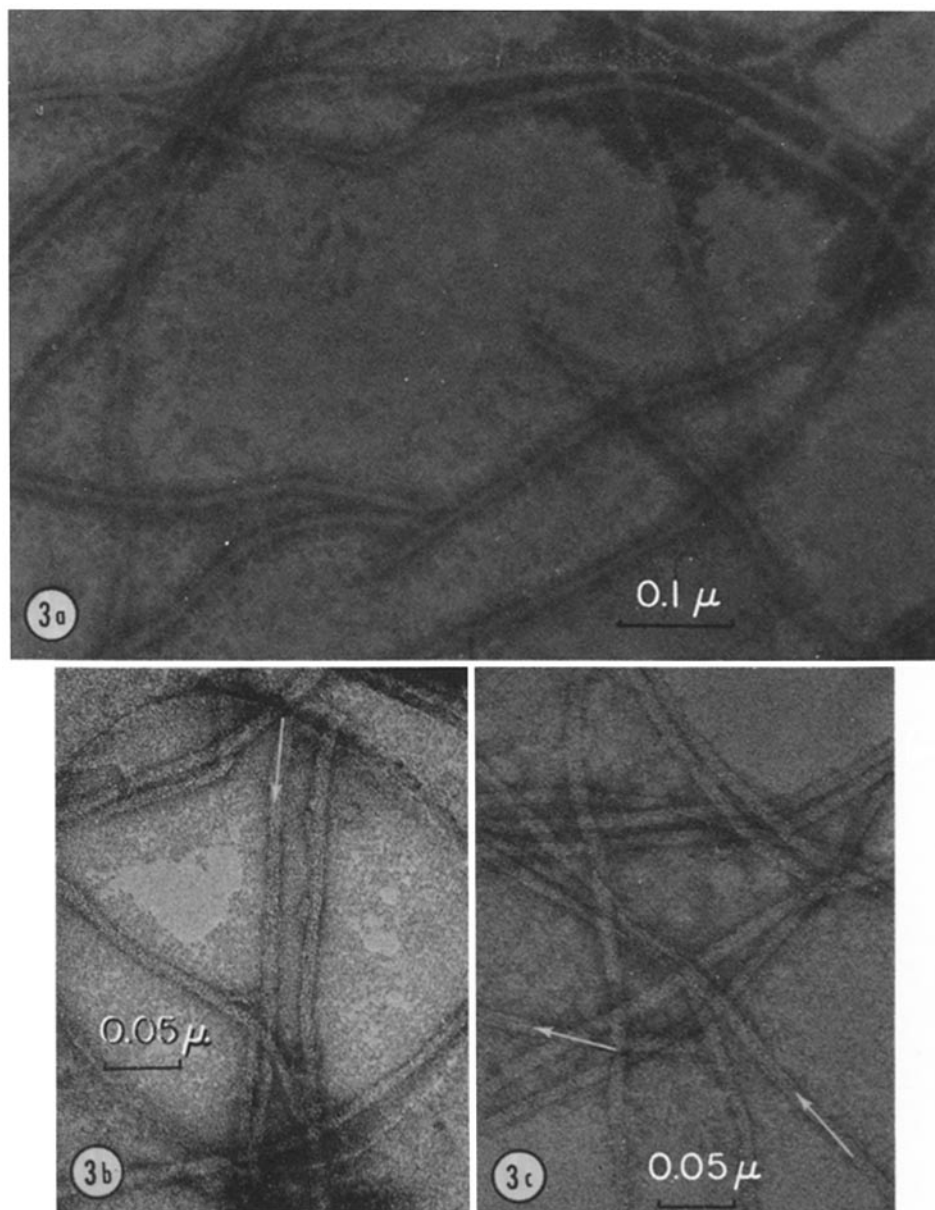


FIGURE 3a Same specimen as Fig. 2b. Note that the ends of the microfibrils do not show any subdivision. PTA, pH 3.0. $\times 152,000$.

FIGURE 3b Dispersed cortical material from rat vibrissal follicles treated with 0.1% chymotrypsin negatively stained with uranyl acetate. Note the clarity of the staining where the fibril is supported by stain alone. The stain is granular but there is no evidence of structural regularity along the microfibrils. A slight increase in density along the center of the microfibrils is evident (arrowed). $\times 218,000$.

FIGURE 3c Preparation as in Fig. 3b negatively stained with uranyl formate. Arrows indicate a slight increase in density along the center of the microfibrils. $\times 214,000$.

Isolated microfibrils did not show any significant regular structure, even when supported by negative stain alone (11) (Fig. 3b). In some regions a slight increase in density along the center of the filaments (arrowed in Figs. 3b and 3c) might be indicative of a somewhat irregular penetration of stain to reveal a central core. Examination of the terminal ends of the microfibrils (Fig. 3a) does not reveal any further subdivision and there was no evidence of filaments of smaller diameter in any of the samples examined.

DISCUSSION

The fibrillar material obtained from rat vibrissal follicles by mild treatment with enzyme was in great abundance and nearly always in close association with the long, easily recognizable bundles of macrofibrils. These morphological features indicate that the fibrils originated from cortical, and not inner-root sheath, cells. Since the use of ultrasonics has been avoided it is most unlikely that any filamentous material could result from the presence of cellulose contamination (17). It is, therefore, reasonable to assume that the isolated filaments do in fact correspond to the microfibrils observed in cross-sections of the fully keratinized fiber. Furthermore, there is excellent agreement between the diameter of the individual fibrils and the values obtained from previous electron microscope data (6, 8, 19). It is perhaps relevant to note that bacterial flagella (15) are similar in appearance to the above structures but are generally greater in diameter (10–20 nm).

The wealth of meridional reflections in the X-ray pattern of α -keratin indicates considerable structural regularity along the fiber axis, but the microfibrils and also the macrofibrils showed no periodic structure when stained with either uranyl or phosphotungstate complex-ions. This observation is perhaps surprising in view of the results obtained with tropomyosin (2) and collagen (14) where the molecules aggregate to form tactoids or fibrils exhibiting characteristic banding patterns when suitably stained. It might, therefore, be concluded that the aggregation of keratin molecules is such that there is an inadequate frequency of acidic and basic staining sites for a periodicity to be detected in the individual microfibrils. However, the possibility remains that, if adequate penetration of suitable metal ions into the macrofibrils could be obtained, then structural regularity might be revealed in the electron image.

Further corroborative evidence that the fila-

ments isolated are indeed microfibrils is provided by the indication, if only slight, of limited penetration of stain to reveal a central core, which is compatible with the high resolution electron images obtained from cross-sections (8, 18) and also with the picture of the microfibril which has been synthesized from the low-angle equatorial X-ray reflections (9).

The regular appearance of the isolated microfibrils indicates that the chymotrypsin has preferentially attacked, and probably caused the dissolution of, the matrix proteins. The very nature of the microfibril structure, in which the protein chains are mostly in the α -helical configuration, would convey resistance to rapid attack by the enzyme (4).

SUMMARY

The keratogenous zone of rat vibrissal follicles treated with 0.1% chymotrypsin solution has yielded an abundance of dispersed cortical material. Electron microscope examination of the negatively-stained dispersions showed the presence of microfibrils, 7.2 nm in diameter and of considerable length. Neither subdivision nor periodic axial substructure was observed during this preliminary study.

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