THE SIZE OF THE CELLULOSE MICROFIBRIL

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ABSTRACT

Recently the lateral width of the cellulose microfibril has been estimated as 30 A rather than about 150 to 200 A, by extrapolation of data from model shadowing experiments. The difference was attributed to a layer of metal deposited during shadowing. However, direct photographs of the same microfibrils parallel and perpendicular to the direction of shadowing, of unshadowed portions of microfibrils compared with shadowed portions of the same microfibrils, of silver-stained unshadowed microfibrils, and of unshadowed, unstained segments of microfibrils give no evidence of a layer of metal of this thickness in material shadowed under normal conditions. Furthermore, the evidence for microfibril strands of about 35 A in width from negative-staining experiments is subject to a bias from the form of the filaments and from variable positive adsorption of phosphotungstic acid by cellulose. Consequently, the conclusion that the true lateral width of native cellulose microfibrils is about one-fifth of the presently accepted value is not yet justified by unequivocal direct experimental evidence.

Recently Ohad, Danon, and Hestrin (7) have published evidence from metal-shadowing experiments which criticizes the presently accepted size range for the lateral width of cellulose microfibrils (150 to 200 A). Extrapolating from the results of model shadowing experiments by Hall (4), they conclude that "after correcting for a large part probably contributed by deposited metal in the observed width of the microfibrils, the real width is estimated roughly to be in the neighbourhood of 30 A." If their analysis were accepted as correct it would follow that the diameter of cellulose microfibrils (and the dimensions of other small shadowed objects) has been grossly exaggerated in the past. However, it is the purpose of the present paper to show that: (*a*) Metal shadowing, as commonly practised, need not lead to the erroneous conclusion they suspect; (*b*) Even if metal shadowing is not used to enhance contrast, direct electron microscopy of cellulose microfibrils from several sources confirms the presently accepted size range deduced from experiments employing shadowing; and (*c*) In the absence of published evidence to the contrary, recent estimates of the width of cellulose microfibrils, from negative staining, may be too low by an indeterminate amount.

MATERIALS AND METHODS

Specimen Preparation

The washed cells of *Acetobacter xylinum* were obtained essentially as described by Hestrin and Schramm (5) except that the cells were stored at 5°C in phosphate-citrate buffer, 0.01 M in phosphate, 0.003 M in citrate, pH 6.0. Microfibrils were produced in drops on Formvar film by a technique which was described previously (2). This procedure ensures that all water-soluble substances which can dialyze through thin Formvar films are removed prior to drying and mounting of the specimen.

Shadowing Methods

The standard shadowing procedure used in this laboratory is as follows. 4 cm of gold-palladium (60–40) wire, (0.010 inches in diameter, weight 34 mg) are melted electrically to form a bead at the apex of an upright V in a 0.020 inch tungsten wire. After 2

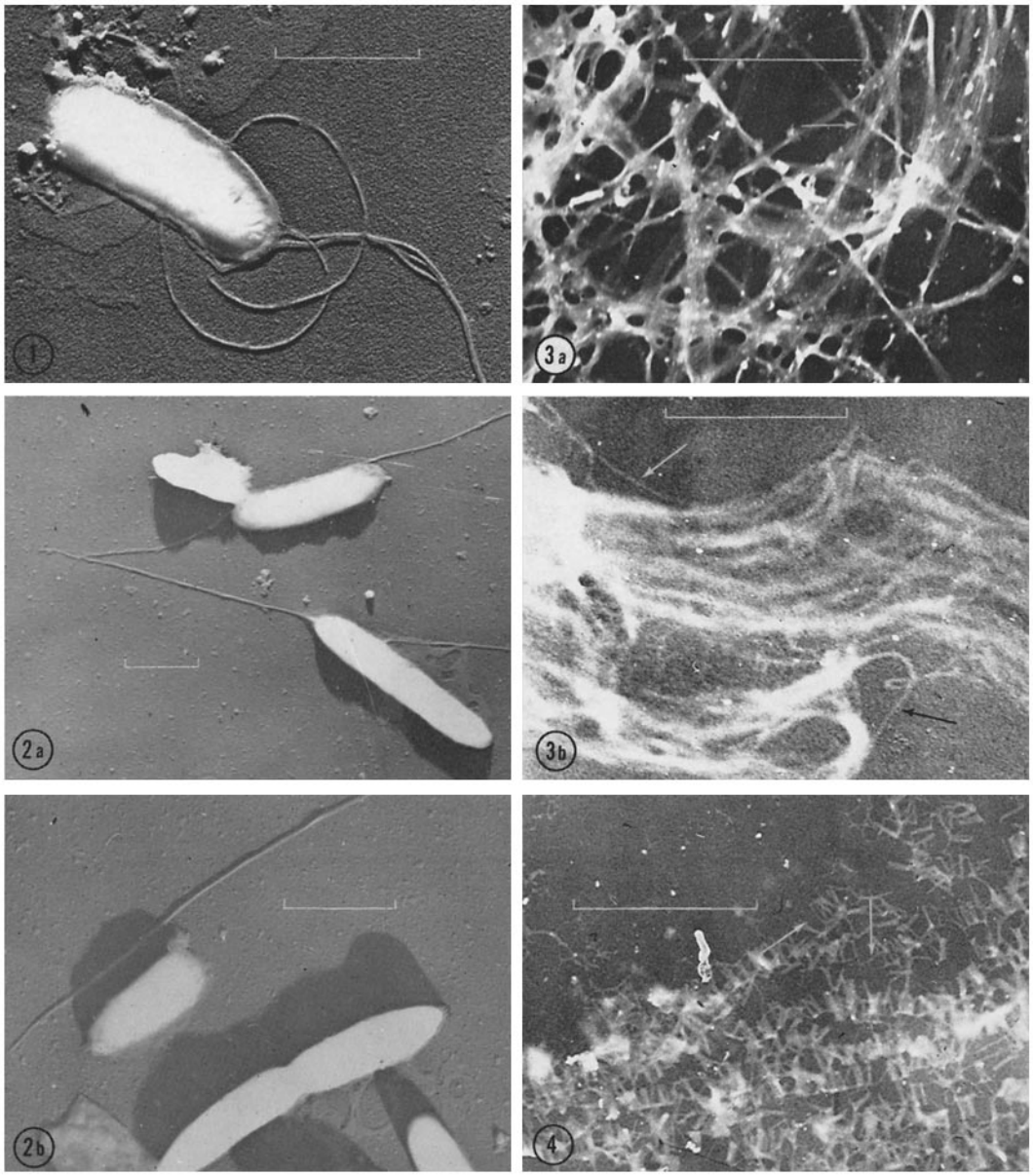

FIGURE 1

Shadowed cellulose microfibrils and a cell of *Acetobacter xylinum*.

FIGURE 2

Shadowed bacterial cellulose microfibrils which pass partly behind bacterial cells. The inferior quality ("muddiness") of both photographs is a result of the necessarily high contrast differences between the shadowed and unshadowed portions. Note the same diameter in both shadowed and unshadowed portions. Also note that all three strands (Figs. 2*a* and 2*b*) are double; *i.e.*, two microfibrils lying parallel.

hours the bead is evaporated at a distance of approximately 20 cm from the specimen to form the metal shadows. As in all shadowing procedures, because of unavoidable asymmetries in the geometry of the apparatus, variations in heating rate, pressure, bead shape, and outgassing from the melted bead, the amount of metal evaporated per unit solid angle is highly variable, both within and between experiments. This variability of metal evaporated per unit angle is probably underestimated in most investigations and, coupled with the unavoidable variations in local angle of deposition on the films, makes any extrapolation from standard model experiments uncertain.

Direct Staining of Microfibrils with Silver Nitrate

Cellulose microfibrils were placed in contact with 5 per cent ammoniacal silver nitrate for 30 minutes, washed, dispersed in a Waring Blendor and mounted on carbon films for examination in the electron microscope.

Direct Microscopy of Unshadowed Segments of Coleoptile Microfibrils

Sections of pectinase-treated coleoptiles were fixed and embedded as previously described (1). Thin sections were cut from the embedded tissue and collected on grids covered with a carbon film. The films and sections were then subjected to ammoniacal silver nitrate for 30 minutes at room temperature, washed in water, and then extracted with ethylene dichloride for 1 hour to dissolve the polymerized methacrylate. The unshadowed residues from the sections were then examined directly in the electron microscope. Because the segments were treated with silver nitrate prior to removal of the methacrylate, the tips may be stained with silver but the remainder of the segment is free from electron-opaque material.

RESULTS

If Ohad, Danon, and Hestrin's conclusion that approximately four-fifths of the apparent width of native cellulose microfibrils is due to a coating of deposited metal were correct, then with microfibrils bent into a circle there would be a noticeable diminution in apparent width as one passes from portions perpendicular to the direction of shadowing to portions parallel to the shadowing. This is because the apparent width of a filament should approach its true width as the axis of the filament approaches parallelism with the shadow direction. However, no such marked diminution occurs (Fig. 1). Although, as expected, the degree of contrast is usually greater when a portion of the microfibril is perpendicular to the direction of shadowing, the apparent width of the portions perpendicular to the shadow is the same as that of the portions parallel to the direction of shadowing. Since in the latter portions the metal coat cannot contribute to the apparent width, Fig. 1 shows that normal shadowing does not grossly distort the estimate of the diameter of the microfibrils, even when enhancing contrast. The estimated width of the microfibrils in the circle is about 200 to 250 A both perpendicular and parallel to shadowing. The same conclusions may be drawn from the oval shaped microfibril in the lower left hand corner of Fig. 3 of Ohad, Danon, and Hestrin (7).

Furthermore, if 80 per cent of the apparent width of native cellulose microfibrils, as commonly observed, were due to metal shadowing, then an abrupt decrease in the apparent width should be observed when the microfibril(s) passes into the shadow of a larger object such as a whole cell. This is because the apparent width of the microfibril clearly cannot be distorted by metal in the unshadowed portion. Such a decrease should be of the order of fivefold and therefore easily detected. However, no such decrease occurs (Fig. 2). The contrast necessarily falls abruptly but the apparent width of the microfibrils does not de-

FIGURE 3*a*

Silver-stained bacterial cellulose microfibrils. The diameters listed in the text refer to the finest resolvable strands.

FIGURE 3*b*

Silver-stained pine holocellulose microfibrils without shadowing.

FIGURE 4

Unstained, unshadowed segments of *Avena* coleoptile microfibrils.

crease detectably when the image passes from the shadowed to the unshadowed portions of the specimen. Here too, both portions of individual microfibrils are about 200 A in width.

Although shadowing of cellulose microfibrils is practiced almost universally in order to enhance contrast it is possible to study these and other fibrillar materials without this aid. Fig. 3 shows electron micrographs of both bacterial cellulose microfibrils and pine microfibrils, stained with silver nitrate, but unshadowed. Contrast is unavoidably poor but the single microfibrils in both samples have an apparent diameter of about 150 to 200 A. In addition, Fig. 4 shows direct electron micrographs of unshadowed, unstained segments of single *Avena* coleoptile microfibrils. The contrast is inadequate but once again the lateral width of these segments of single microfibrils, which are unshadowed, is about 180 A.

DISCUSSION

The above results show that when metal shadowing is used to enhance contrast of native cellulose microfibrils in electron microscopy the resulting sheath or cap of deposited metal need not lead to gross errors in the estimate of their width. Moreover, single cellulose microfibrils or segments of microfibrils may be resolved without any shadowing and the resulting images are of the same order of size as the dimensions deduced from shadowed specimens. Clearly, therefore, although it is well known that the metal cap adds somewhat to the size of small objects (3), this addition need not be 4 to 5 times their original dimension under ordinary conditions of shadowing.

Recently, results from negative staining have also indicated that the size of cellulose microfibrils may have been overestimated. Photographs of material examined by this method have suggested that the diameter of the finest threads in cellulose microfibrils is about 35 A (6). However, although this technique has the apparent advantage of leaving the microfibrils in their original condition it may introduce a bias towards minimizing the width of strands such as cellulose microfibrils. The basis of this bias is illustrated in Fig. 5. Since the electron scattering material is used to outline the specimen, if the shape and size of the object permits encroachment of the electron-opaque compound, the apparent edge of the object may be moved inwards. Therefore, depending on the relative thicknesses of the electron absorbing layer and the diameter of circular or oval objects under examination, the apparent width may vary below the true width. Errors of this nature might be avoided by comparison of the shape of a microdensitometer trace across images of the objects with calculated shapes, but this is seldom done and has not yet been reported for cellulose microfibrils.

In addition, the use of the negative staining technique assumes little or no adsorption of the electron-opaque material by the object under examination. However, experiments in our laboratory have shown that for phosphotungstic acid and cellulose microfibrils this assumption is not always valid. For reasons which are not yet understood, phosphotungstic acid may sometimes be adsorbed strongly to native cellulose microfibrils, thereby vitiating the purpose of the experiment. If, as seems likely, this adsorption is predominantly due to the paracrystalline outer sheath of the microfibril and not to the crystalline core, there would be a marked decrease in the apparent width of the microfibrils observed by this technique.

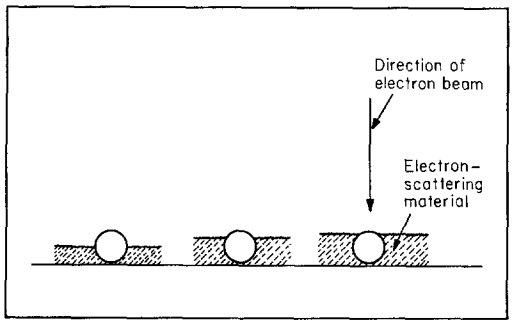

FIGURE 5

Illustration of source of possible bias in estimating minimum dimensions of small objects using the negative-staining technique.

The above photographs together with consideration of the phosphotungstic acid technique illustrate that there is as yet no unequivocal experimental evidence for the view that cellulose microfibrils are only about one-fifth the presently accepted diameter. Extrapolation from metal shadowing experiments may well be less justified than accepting the direct unequivocal images. Sources of error in this extrapolation involve at least the following points: (*a*) large variations in geometrical factors (the socalled *x* factor); (*b*) large

and unavoidable variations in local film curvature; and (*c*) large and unavoidable variations in density of deposition of metal shadow from experiment to experiment.

These factors preclude any safe extrapolation from one group of results to another without the application of the most stringent caution.

Although the above results illustrate that the diameter of cellulose microfibrils may still be accepted as 150 to 200 A, they do not imply that real differences in microfibril size do not exist both within and between species (7). This is a separate, important problem which has not yet received the intense careful study it deserves. The additional related problem of the detailed mechanism of formation of the microfibril unit will be considered elsewhere.

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